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Evaluation of the Human/Extreme Environment Interaction: Implications for Enhancing
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14. ABSTRACT

The purpose of this research determines how hypoxia interacts with exercise and recovery to yield various metabolic responses that may affect performance and performance at high-altitude critical to mission success. Participants completed a maximal exercise test under normoxic conditions (975 m). Four experimental trials were completed including 60 minutes of cycling (70% of Wmax) followed by 6 h of recovery at four different simulated altitudes (0 m, 1667 m, 3333 m, or 5000 m) using a randomized, counterbalanced, cross-over design over the span of 4 weeks, with a minimum of 7 days between trials. Blood O2 saturation was measured via pulse oximetry every hour during the 6 h recovery period. Muscle biopsies were obtained from the vastus lateralis pre- and 6 h post-exercise for analysis of mitochondrial related gene expression. Blood O2 saturation decreased with each increase in simulated altitude during recovery (0 m: 98 ± 1%; 1667 m: 94 ± 1%; 3333 m: 90 ± 1%; 5000 m: 79 ± 2%; $p < 0.05$). Expression of PGC-1α, HK, and SOD increased significantly with exercise ($p < 0.05$), but were not different between trials. Similarly, markers of oxidative stress indicated a time-dependent response following exercise for all variables ($p<0.05$), but the two highest recovery altitudes (3333m and 5000m) demonstrated a partially attenuated this response for LOOH ($p<0.001$). These data demonstrate no dose-response relationship between magnitude of hypoxic exposure and mitochondrial gene expression yet minor alterations in oxidative stress. Therefore, the paradox of mitochondrial function in response to acute and chronic exposure to hypoxia cannot be explained by the magnitude of hypoxia. Of additional interest in year three of this project series related to the evaluation of advanced instrumentation to determine changes in hydration status in parallel to established markers from urine and isotopic enrichment methodologies. Using an aggressive field research model we have developed, hydration parameters relative to real-time physiological monitoring metrics and gold-standard measures of total energy expenditure (TEE) were evaluated. With average measures of TEE of 19.1±3.9 MJ/day and rates of water turnover averaging (9.5±1.7 L/day), an aggressive work environment was demonstrated. Despite a wide range of responses from mobile physiological monitors, we were unable to establish a predictive model to quantify hydration demands from advanced modeling of physiological metrics because of multiple instrumentation errors. If real-time physiological monitoring to evaluate heat and physiological stress, instrumentation capabilities require re-engineering to minimize data loss. Despite monitor failures, we have more firmly established the potential of skin temperature measurements to provide real-time heat stress awareness. Overall, this research series suggests that the role of intermittent simulated altitude exposure (via normobaric hypoxia) should be reconsidered as an approach to attempt to acclimate individuals for the operational environment. Our data demonstrates an apparent disruption in the mitochondrial gene response post exercise when normobaric hypoxia is utilized during the recovery. More research regarding the effects of overnight exposure periods is warranted (as in the use of hypoxic tents for sleeping). Of additional interest, the field research efforts suggest that the wildland firefighter (WLFF) research model provides an ideal parallel to the warfighter. As we have previously demonstrated, the operational environment of the WLFF is similar to the training continuum and operational environment of the warfighter. However, our data also demonstrates that if mobile physiological monitoring is warranted, the engineering and design of these systems requires serious reconsideration. Monitoring systems should strive to incorporate meaningful metrics that allow for real-time data access so that adequate decisions can be made throughout the training continuum and the operational environment.

15. SUBJECT TERMS- hypoxia, exercise recovery, oxidative stress, metabolic genes, mitochondrial genes, total energy expenditure, water turnover, physiological monitoring.

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INTRODUCTION:

Highly trained military forces must perform at peak performance during combat operations. The main goals of this proposal have been to measure changes in muscle cells after exercise in cold and high altitude environments. Because the stresses of hard work can affect how well a soldier may perform on the next mission/assignment, we are interested in how the environmental stress and selected nutrition may interact during recovery. This research plan uses methods that have direct application to the soldier's operational environment. The altitude stress included has direct relevance to the current conflicts and U.S. troop presence in Afghanistan.

These studies have been developed to determine the effects of harsh environments on the body and what types of pre-mission training and what types of mission nutrition can improve performance, safety and recovery of the soldier. The application of these results should be used during early training of soldiers to increase the muscles adaptation(s) to training. During this second year of the project series our specific aim was to determine the impact of exercise and recovery under hypoxic stress on metabolic genes and oxidative stress markers. These data build of our findings from year 1 by advancing our understanding of how acute the exercise stress may interact with environmental conditions to alter the responsiveness of the skeletal muscle. These data have further implications on training and environmental exposure strategies and how they could be used to enhance mission preparedness.

In conjunction with our laboratory efforts, an aggressive field study was designed to evaluate the effectiveness of advanced physiological monitoring systems to accurately estimate hydration and energy expenditure during arduous, hot work conditions. Previously, our research team has utilized the working efforts of the US Forest Service Type I Interagency wildland firefighter. We have demonstrated the hydration demands, energy expenditure of the job and the implications of supplemental feeding in this population over the last several years. It was anticipated that physiological metrics would align with gold standard measures of water turnover and TEE from isotopic dilution to develop real-time metrics associated with hydration demands.

BODY:

The central scientific question from this research revolves around how hypoxia interacts with exercise and recovery to yield various metabolic adaptations that may affect performance and performance at high-altitudes specific to the operational environment. The process or our work has been outlined below relative to our original statement of work aligned with year 3. Throughout the project, we have remained on schedule or slightly ahead of schedule and are currently in the process of finalizing our findings from year 3. Additionally, despite the unpredictable nature of wildland fire activity, all the field data collection was completed on time.

Year 3

Task 7. Data collection for lab study 3 (months 19-22).

7a. Revise approved IRB protocols for any necessary changes suggested by laboratory study 1 and 2 and recruit subjects (month 19).

The third large-scale laboratory study was approved by the University of Montana IRB in November, 2012 and submitted for HRPO review on November 20, 2012. Initial approval was established by HRPO on November 21, 2012 with continuing review acceptance on January 15, 2013.

7b. Data collection for laboratory study 3 (months 19-22).

Initial equipment testing and preliminary study participant testing was initiated on Data collection for the third laboratory study was initiated on January 22. The initial hypoxia trials were initiated on January 28, 2013. The final data collection efforts for all hypoxia trials (4 trials each subject) were completed on March 28, 2013, as originally planned. What is especially important to note is that during data collection, we achieved 100% compliance and with all of the research study participants completing the protocol as originally scheduled. We did not have to reschedule any of the $4 \times 12 = 48$ trials due to sickness or schedule conflicts. We only had data collection issues on the first hyperoxic trial with the metabolic system in the chamber, which was remedied for all other subsequent trials.

Task 8. Data analyses for laboratory study 3 (months 21-24).

This has been completed in addition to all statistical analyses.

8a. Order necessary analytical kits, probes, primers and other analyses needs (month 21).

This has been completed.

8b. Finalize analytical techniques and analyze all samples (months 22-23).

This has been completed.

8c. Data analyses of descriptive and other data collected (non biochemical) (months 23).

This has been completed.

8d. Statistical analyses of all data (months 23-24).

This has been completed.

Task 9. Data summaries and manuscript preparations (months 24-36)

9a. Compile all statistical results and data analyses to complete manuscripts from laboratory studies 1-3.

This has been completed.

Task 10. Complete field study (months 30-36).

10a. Test trials for equipment to be used during laboratory (months 24-26).

This has been completed.

10b. Data collection for field study will require testing during months 34 and 35 due to the summer fire season months.

This has been completed in conjunction with other data collected during the fire season of year 2.

10c. Data analyses for field study (months 34-35).

This has been completed.

Findings from Year 1 – Laboratory Study Series

Introduction

It has been largely assumed since 1962 that hypoxic conditions and/or altitude stimulate muscle oxidative capacity. This notion comes from the observation that active Peruvian miners had 78% more cytochrome c reductase and 16% more myoglobin than low land controls (56). Five years later it was noted that endurance exercise was a potent stimulator of mitochondrial enzymes (29). The data from these two studies formed the consensus that muscle hypoxia (from exercise or environment) was an important stimulus for mitochondrial development. In the early 1990's this tenant was challenged by data before and after mountaineer expeditions to the Himalayas. The findings of these studies demonstrated a loss of muscle cross sectional area, decreased mitochondrial volume, and decreased maximal aerobic capacity (20,31,32). Additionally, after return from these expeditions lipofuscin, a mitochondrial breakdown by-product, was increased (44). These findings of reduced oxidative capacity were further confirmed by "Expedition Everest II" (42). Low levels of mitochondrial capacity have also been shown in the high altitude residents of La Paz (18) and in Sherpa's (37). Paradoxically, these high land populations have excellent physical performance at altitude.

When exercise training under hypoxic conditions, but recovering in a normoxic environment, a slightly different story emerges (train high, live low). When this type of short-term exposure is incorporated into a training paradigm mitochondrial density, maximum aerobic capacity, citrate synthase activity, and anaerobic performance is enhanced over normoxic control exercise (5,17,23,27,46,47,63,64). Functionally, increased glucose metabolism shown with altitude exposure and acclimatization (12) may be more efficient in that this adaptation would lead to more ATP per molecule of oxygen. The paradox with short-term and long-term metabolic adaptations is yet to be completely understood, however it may be related to the recovery process and specifically indications of decreased protein synthesis during hypoxia (26).

The mechanisms to which hypoxic adaptations occur are even less clear, but seem to be regulated by hypoxia-inducible factor 1 (HIF-1) (59). HIF-1 is a transcription factor that stabilizes in the nucleus upon exposure to hypoxic conditions (35,62) and in turn induces the expression of hypoxia-induced genes. HIF-1 causes increased gene expression for glucose transporters, glycolytic enzymes, angiogenic factor, and erythropoietin by targeting the hypoxic response element in the promoter region (52,59). Higher HIF-1 mRNA levels after training (64,69) may produce an increased potential for quick activation of HIF-1 with the onset of hypoxic stimuli after training at altitude (30). However, the response of HIF-1 after exercise training is blunted in response to an absolute intensity exercise protocol (41). Thus, HIF-1 stabilization may be increased by exercise and factors independent of hypoxia. Indeed, interleukin-1beta, insulin like growth factor I and II, insulin, heregulin, epidermal growth factor TNF alpha, angiotensin-2, and nitric oxide have been shown to be capable of inducing HIF-1 (19,25,28,39,57,68). The complex nature of HIF-1 activation serves a common endpoint to delivery oxygen and up-regulate the metabolic machinery of the cell.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) gene expression is strongly induced by exercise (2,38,50) contributing to mitochondrial biogenesis and metabolic alterations. Recent data from our lab has shown PGC-1α mRNA to increase with acute exercise, but have a blunted response after 11 and 21 days of endurance training (Figure 1). PGC-1α expression is also induced by hypoxia (1) and does not appear to be dependent on HIF-1. The activation of PGC-1α stimulates mitochondrial biogenesis and hence contributes to increased fatty acid fuel oxidation and subsequent exercise performance (40,66).

The current view of the mitochondria is not one of many mitochondria, but of a single mitochondrion within a cell where the solitary shapes of mitochondria are considered to be sections of mitochondrial tubules. Fusion (growth and development) of these tubules has been shown in healthy conditions such as exercise (6) and weight loss (22,48) while fission (degradation, dysfunction) is associated with obesity and disease (3,4). We have recently observed an increase in Mitofusin 2 (MFN2) mRNA a mitochondrial fusion gene with acute exercise throughout 21 days of endurance training. It has been suggested that the balance between fusion and fission may play a central role in the metabolic consequences of hypoxia (36).

These two distinct pathways of metabolic alteration with hypoxia (1. HIF-1 and 2. PGC-1α) have very different effects. The HIF-1 pathway leads to increased reliance on carbohydrate substrates and the PGC-1α pathway leads to increased reliance on fat substrates. Clearly, investigations are needed to discern the pathways and applied metabolic outcomes associated with hypoxia. This information will allow for protocol development for not only altitude/hypoxia tolerance, but also for exercise performance, both of which may be critical to mission success in harsh environments.

Exercise intensities during exercise studies are usually determined by incorporating intensity relative to maximal aerobic capacity (i.e. 60% VO2 max). This becomes a challenge when studying the effects of hypoxia and altitude versus normoxic control conditions because acute hypoxia decreases VO2 max when compared to normoxic conditions (16,21,24,43,53,58,61,67). Endurance trained individuals with an average sea level VO2 max of 65.5 ml/kg/min will have an average VO2 max at 2500 m of 57.7 ml/kg/min, a 7.8% decrease (49). When exercise intensity is set at 65% of environmental VO2 max (as is typically done) the absolute exercise intensities will differ in that the hypoxic intensity will be at a lower absolute intensity (42.58 ml/kg/min under normoxic conditions and 37.51 ml/kg/min under hypoxic conditions). Thus difference in absolute intensity may be a contributing factor (other than hypoxia) to studies incorporating this method of choosing exercise intensity. Therefore, the purpose of this project was to determine the impact on metabolic genes in response to exercising at an intensity relative to hypoxic VO2 max in hypoxic conditions, relative to normoxic VO2 max in hypoxic conditions, and relative to normoxic VO2 max in normoxic conditions.

METHODS

Participants

Twelve male participants (24 ± 1 yrs, 183 ± 2 cm, 82 ± 3 kg, 4.2 ± 0.3 L•min^{-1} (975 m), 4.0 ± 0.2 L•min^{-1} (3000 m)) completed the study. Subjects completed a Physical Activity Readiness Questionnaire (PAR-Q) and were briefed on the experimental protocol and possible risks prior to giving written informed consent. All procedures were approved by the University Institutional Review Board (The University of Montana, Missoula, MT).

Preliminary Testing
Body composition was measured using hydrodensitometry. Underwater mass was measured with a digital scale (Exertech, Dreshbach, MN). Body Density was corrected for estimated residual lung volume (10) and converted to percent body fat using the Siri equation (60). Two graded maximal exercise tests (starting at 95 W, and increasing 35 W every 3 minutes) were completed (minimum 2 days between tests) on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) to determine maximal aerobic capacity (VO$_{2max}$) and the power output associated with VO$_2$max (W$_{max}$) at 975 m and 3000 m. Expired gases were collected and analyzed during the test, using a calibrated metabolic cart (ParvoMedics, Inc., Salt Lake City, UT). VO$_{2max}$ was assigned to the highest achieved oxygen uptake recorded during the test. W$_{max}$ was calculated by adding the Watts in the last completed stage to the fraction of time spent in the uncompleted stage multiplied by 35.

Experimental Protocol
Design. Participants completed 3 trials [one exercise trial in the simulated altitude environment at an intensity of 60% of their hypoxic peak power (HH); one exercise trial in the normal lab altitude at an intensity of 60% of their hypoxic peak power (NH); and one exercise trial in the normal lab altitude at an intensity of 60% of their normoxic peak power (NN)] using a randomized, counterbalanced cross-over design over the span of 3 weeks, with a minimum of 7 days between trials. All trials were completed in a temperature, humidity, and hypoxia (Colorado Altitude Training, Louisville, CO) controlled environmental chamber (Tescor, Warminster, PA) in ambient conditions of 12°C and 40% relative humidity. Participants kept an exercise record for 2 days before and a dietary record for 24 h before the initial trial and replicated exercise and diet for these periods before the remaining trials. Additionally, participants abstained from exercise 24 hours before each trial. Following an overnight 12 hour fast, participants arrived at the laboratory in the early morning to complete testing. Upon arrival to the laboratory nude body mass was measured (CW-11, Ohaus Corporation, Pine Brook, NJ). The trials consisted of 1) cycling for 1 hour at 60% of peak power (as measured at 975 m) at 975 m and recovering at 975 m for 4 hours, 2) cycling for 1 hour at 60% of peak power (as measured at 3000 m) at 975 m and recovering at 975 m for 4 hours, and 3) cycling for 1 hour at 60% of peak power (as measured at 3000 m) at 3000 m and recovering at 3000 m for 4 hours. During the recovery period, participants changed out of their cycling clothes, toweled off, and wore a standardized sweat suit to mitigate shivering. Participants remained in a sitting position throughout the 4 hour recovery period. Nude body mass was measured following the ride and at the end of the 4 hour recovery period. Participants consumed 8 ml•kg•min^{-1} during the ride and 8 ml•kg•min^{-1} during the recovery.

Biopsies. Muscle biopsies were taken before exercise and at the end of the 4 hour recovery period for each trial. Biopsies were taken from the *vastus lateralis* muscle using a 4-5 mm Bergstrom percutaneous muscle biopsy needle with the aid of suction (8). All subsequent

biopsies during a given trial were obtained from the same leg using a separate incision 2 cm proximal to the previous biopsy. After excess blood, connective tissue, and fat were removed, tissue samples were stored in RNA Later or immersed in liquid nitrogen and stored at -80°C for later analysis.

Blood Samples. A 10 mL and 6 mL blood sample was taken from an antecubital vein before exercise and at 0, 2, and 4 hours during recovery. Immediately, 2 capillary tubes were filled with 100ul of blood each and placed in a hematocrit centrifuge for 3 minutes, 15,290 x g (A13, Jouan, Winchester, VA) for analysis of hematocrit. The proportion of blood cells to serum was measured in each tube and the average of the two measures was used to determine hematocrit. The remainder of the blood sample was kept at room temperature for 20 minutes to allow clotting. Once clotted the samples were centrifuged at 7500 x g for 20 minutes (MR 22i, Jouan, Winchester, VA). Serum was removed and stored at -80˚C until subsequent analyses.

Respiratory Parameters. Expired gases were collected during exercise at 0, 27.5, and 53 min of exercise to evaluate differences in oxygen consumption and respiratory function among trials. Each collection was collected for 5 min, with the last 2 min averaged to represent the sample period.

Pulse Oximetry. Blood oxygen saturation was evaluated pre ride, during the ride at 4, 31.5, and 57 mins, and at 0, 2, and 4 hours during the recovery using a pulse oximeter (Nonin Onyx II 9550, Plymouth, MN).

Analysis

Skeletal Muscle RNA isolation. An 8-20 mg piece of skeletal muscle will be homogenized in 800ul of Trizol using an electric homogenizer. The samples are then incubated at room temperature for 5 minutes after which 200ul of chloroform per 1000ul of Trizol is added and shaken vigorously by hand. After an additional incubation at room temperature for 2-3 minutes the samples are centrifuged at 12,000 g for 15 minutes and the aqueous phase was transferred to a fresh tube. The RNA is then precipitated by adding 500ul of isopropyl alcohol pre 1000ul of initial Trizol and incubated overnight at -20°C. The next morning samples are centrifuged at 12,000g for 10 minutes at 4°C and the RNA is washed by removing the supernatant and adding 1000ul of 75% ethanol per 1000ul of initial Trizol. The samples are then mixed by vortex and centrifuged at 7,500g for 5 minutes at 4°C. The RNA is then redissolved in 100 ul RNase-free water after the supernatant is removed and the RNA pellet was dried. The RNA is then cleaned using the RNeasy mini kit (Qiagen) according to the manufactures protocol using the additional DNase digestion step (DNA mini kit, Qiagen). RNA is then quantified using a nano-spectrophotometer.

cDNA synthesis. First-strand cDNA synthesis is achieved using Superscript-fist strand kit (Invitrogen) according to the manufactures protocol. Each sample within a given subject will

contain the same amount of RNA (400 – 1000 ng). The resulting cDNA was then diluted using RNase free water in order to have adequate volume for RT-PCR and frozen for later RT-PCR analysis.

Real time RT-PCR. Each 25ul reaction volume will contain 500nM primers, 250nM probe (PrimeTime qPCR assay, Integrated DNA technologies), 1x FastStart TaqMan Probe master (Roche Applied Science), and 2.5 ul of sample cDNA. PCR will then run using the Bio-Rad I Cycler iQ5 Real-Time PCR Detection system (Bio-Rad) using a 2-step Roche protocol.

Oxidative Stress. Plasma aliquots were assayed for an oxidative stress biomarker panel used previously by our group (33,55). Two biomarkers, plasma trolox-equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP), were chosen to evaluate blood plasma antioxidant capacity. Oxidative damage was evaluated in plasma by determination of protein carbonyls and lipid hydroperoxides content.

Antioxidant Capacity. Plasma antioxidant capacity was measured by the plasma trolox-equivalent antioxidant capacity technique whereby a radical cation of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) reaction is quenched by sample-specific antioxidant fortifications (15). Total plasma antioxidant potential was determined by the ferric reducing antioxidant potential assay according to the methodology of (7). Both TEAC and FRAP assays result in quantifiable colorimetric solutions which are visualized spectrophotometrically. Lipid peroxidation was determined by the ferrous oxidation-xylenol orange assay (51). In brief, ferrous ions are oxidized by lipid hydroperoxides to ferric ions and subsequently react with the ferrous sensitive dye containing xylenol orange. In the presence of lipid hydroperoxides, this reaction forms a spectrophotometrically quantifiable complex. Protein carbonyls were analyzed using a commercially available ELISA kit (Zentech Technology, Dunedin, New Zealand). All assays were performed in triplicate and exhibited within sample coefficients of variation between 2% and 5%. Prior to analysis, all plasma samples were assayed in quadruplicate for protein concentration based on the methods of Bradford et al, (1976) and adjusted to 4 mg/ml protein using a phosphate buffer (11). All oxidative stress biomarkers were normalized for plasma volume shifts experienced during the three trials.

Statistics

Muscle glycogen, substrate utilization, mRNA of metabolic genes and oxidative stress markers were analyzed using a repeated measure ANOVA (trial*time). A probability of type I error less than 5% was considered significant ($p<0.05$). All data is reported as means ± SE.
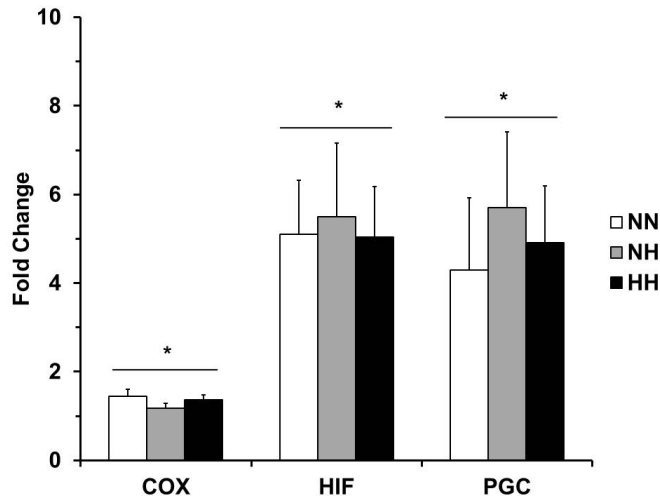

RESULTS:

Glycolysis Genes:
HK and PFK increased as a result of the exercise and recovery ($p<0.001$ and $p<0.001$ respectively) but were not different between trials ($p=0.126$ and $p=0.419$ for HK and PFK, respectively).
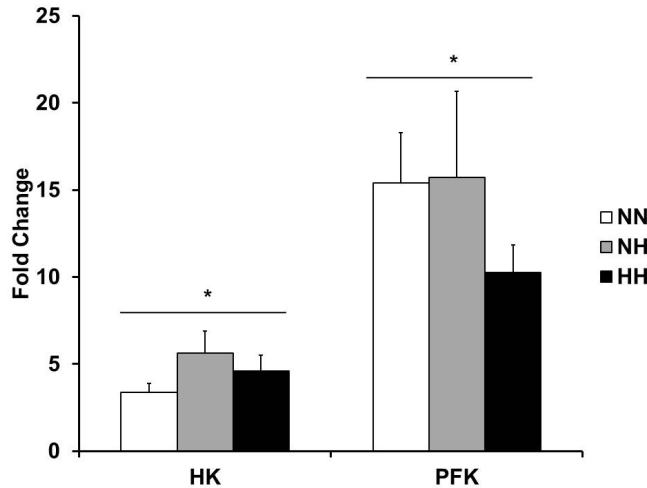
Figure 1. Changes in HK and PFK in response to the three exercise/recovery conditions. *p<0.05, main effect of time. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Metabolic Genes:
COX, HIF, and PGC increased as a result of the exercise and recovery (p<0.001, p=0.002, and p-0.003 respectively) but were not different between trials (p=0.342, p=0.951, and p=0.849 for COX, HIF, and PGC, respectively).

Figure 2. Change in COX, HIF, and PGC in response to the three exercise/recovery conditions. *p<0.05, main effect of time. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

*Mitochondrial Morphology Genes:*

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FIS, and MFN increased as a result of the exercise and recovery (p<0.007 and p=0.001 respectively) but OPA did not (p=0.923). FIS, MFN, and OPA were not different between trials (p=0.368, p=0.374, and p=0.068, respectively).
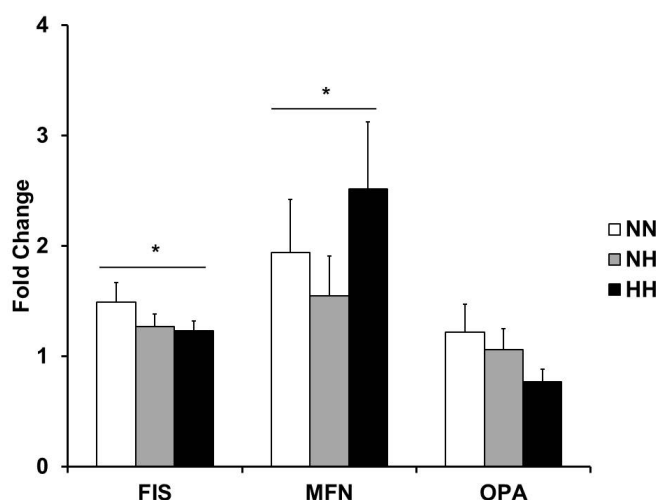
Figure 3. Changes in FIS, MFN, and OPA (*p<0.05 vs. main effect for time) in response to the three exercise/recovery conditions. *p<0.05, main effect of time. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Glycogen:
Muscle glycogen was similar pre exercise between all three trials (p>0.05). Muscle glycogen was lower at 4 h post in the NN trial vs. the NH trial (p=0.030), but was not different among other trials (p>0.05).
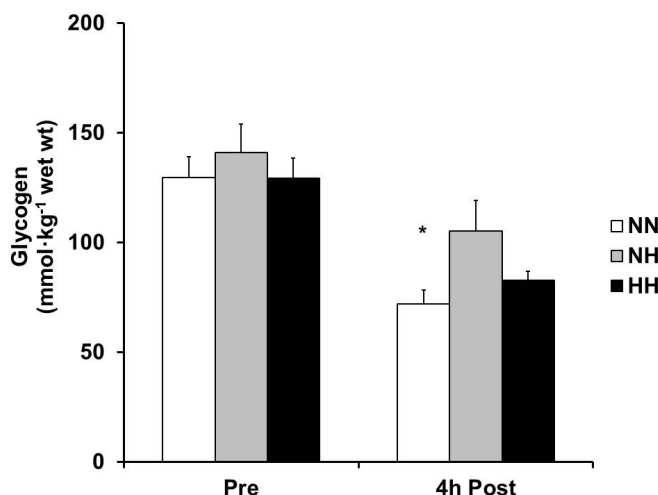
Figure 4. Changes in muscle glycogen in response to the three exercise/recovery conditions. *p<0.05, interaction effect. (NN = normal lab altitude at an intensity of 60% of their normoxic
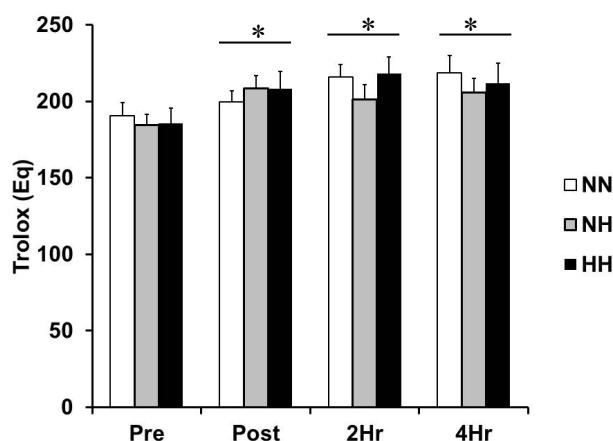
peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Ferric reducing ability of plasma (FRAP): Trial main effects were noted for FRAP (Trial p=0.0138, Time p=0.241), and cannot be explained currently.
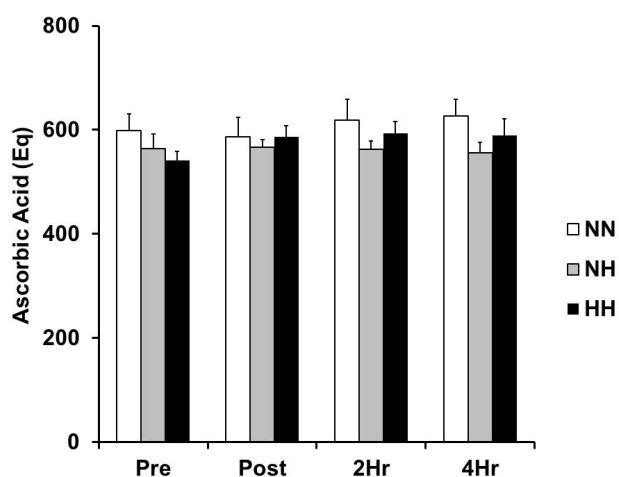
Figure 5. Changes in ferric reducing ability of plasma (FRAP) in response to the three exercise/recovery conditions. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Trolox equivalent antioxidant capacity of plasma (TEAC): Time main effects for TEAC (Trial p = 0.263, Time p<0.0001 (Pre vs. Post, 2Hr, & 4Hr)) likely reflect an increase in circulating plasma urate due to the 3 exercise bouts.

Figure 6. Changes in trolox equivalent antioxidant capacity of plasma (TEAC) in response to the three exercise/recovery conditions. *p<0.05, main effect of time. (NN = normal lab altitude
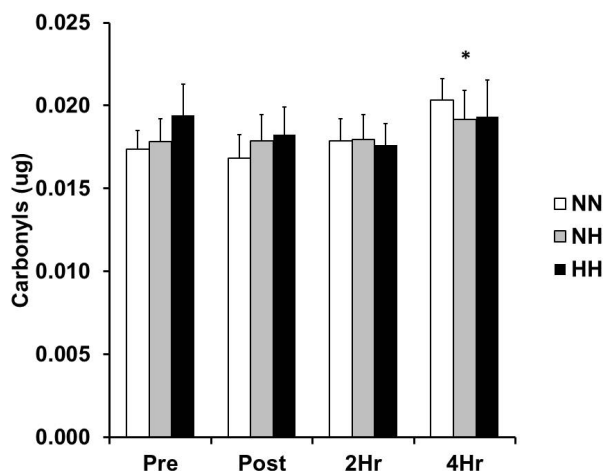
at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Plasma lipid hydroperoxide (LOOH): LOOHs exhibited the most dramatic response to the 3 exercise trials (Main effects: Trial p=0.009, Time p<0.0001) with significant increases being observed following both the high altitude simulation trials. Trial HH elicited an increase in LOOH Post and 2Hr time points, while Trial NN and HH were different at 2Hr. Moreover, NH LOOH values were increased over at 4Hr post exercise as compared to Pre.
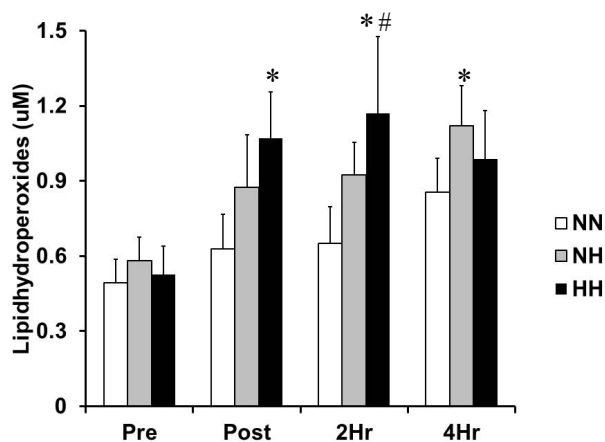
Figure 7. Changes in plasma lipid hydroperoxide (LOOH) in response to the three exercise/recovery conditions. *Different from Pre, main effect of time, p<0.05. #Different from NN, main effect trial, p<0.05. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

Plasma protein carbonyls (PC): A similar Time main effect was noted for PCs, indicating a rise in circulating oxidized proteins following exercise, though no trial main effects were noted (Main effects: Trial p=0.577, Time p=0.012 for 4Hr vs. Post and 2Hr time points).

Figure 8. Changes in plasma protein carbonyls (PC) in response to the three exercise/recovery conditions. *$p<0.05$, main effect of time vs. Post and 2Hr time points. (NN = normal lab altitude at an intensity of 60% of their normoxic peak power; NH = normal lab altitude at an intensity of 60% of their hypoxic peak power; HH = simulated altitude environment at an intensity of 60% of their hypoxic peak power)

SUMMARY

Metabolic Genes

The main finding here is the lack of difference between trials for our select metabolic genes. This is an important finding in that it allows us to optimize future protocols to distinguish a gradient of adaptation between trials. Specifically, we suggest increasing the amount of hypoxia in the simulated high altitude trials. These subtle alterations in the hypoxic environment have been included in the second year methodology and have received IRB/HRPO approval. These methodologies can be reviewed in more detail in Appendix 1.

Oxidative stress markers

For the current experiments we examined four key biomarkers of blood plasma oxidative stress, ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity of plasma (TEAC), lipid hydroperoxide (LOOH), and protein carbonyls (PC). These biomarkers were chosen for their sensitivity to exercise (9,33,34,45,54,55) in addition to the biological pecking order for oxidative stress reactions in complex biological fluids such as blood plasma (13,14,65). This biomarker panel is sensitive to aqueous and lipid phase antioxidants. The TEAC and FRAP assays are uniquely sensitive for urate and vitamin C. These antioxidants account for upwards of 70% of the antioxidant capacity of plasma. Moreover, this biomarker panel is sensitive to protein and non-protein thiol antioxidant capacity.

Date from this study reveal only modest changes in our plasma oxidative stress marker panel. There was a significant main effect for time for TEAC, which likely reflects an increase in circulating plasma urate in response to each of the exercise bouts. A similar main effect for time was noted for PCs, indicating a rise in circulating oxidized proteins following exercise, though no trial main effects were noted. Trial main effects were noted for FRAP, an

observation that cannot be explained currently. LOOHs exhibited the most dramatic response to the exercise trials with significant increases being observed following both the hypoxic trials.

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Findings from Year 2 – Laboratory Study Series

INTRODUCTION

It has been largely assumed since 1962 that hypoxic conditions and/or altitude stimulate muscle oxidative capacity. This notion comes from the observation that active Peruvian miners had 78% more cytochrome c reductase and 16% more myoglobin than low land controls (34). Five years later it was noted that endurance exercise was a potent stimulator of mitochondrial enzymes (16). The data from these two studies formed the consensus that muscle hypoxia (from exercise or environment) was an important stimulus for mitochondrial development. In the early 1990's this tenant was challenged by data before and after mountaineer expeditions to the Himalayas. The findings of these studies demonstrated a loss of muscle cross sectional area, decreased mitochondrial volume, and decreased maximal aerobic capacity (9, 18, 19). Additionally, after return from these expeditions lipofuscin, a mitochondrial breakdown by-product, was increased (28). These findings of reduced oxidative capacity were further confirmed by "Expedition Everest II" (27). Low levels of mitochondrial capacity have also been shown in the high altitude residents of La Paz (7) and in Sherpa's (22). Paradoxically, these high land populations have excellent physical performance at altitude.

When exercise training under hypoxic conditions, but recovering in a normoxic environment, a slightly different story emerges (train high, live low). When this type of short-term exposure is incorporated into a training paradigm mitochondrial density, maximum aerobic capacity, citrate synthase activity, and anaerobic performance is enhanced over normoxic control exercise (4, 6, 11, 14, 29, 30, 39, 40). Functionally, increased glucose metabolism shown with altitude exposure and acclimatization (5) may be more efficient in that this adaptation would lead to more ATP per molecule of oxygen. The paradox with short-term and long-term metabolic adaptations is yet to be completely understood, however it may be related to the recovery process and specifically indications of decreased protein synthesis during hypoxia (13).

The mechanisms to which hypoxic adaptations occur are even less clear, but seem to be regulated by hypoxia-inducible factor 1 (HIF-1) (36). HIF-1 is a transcription factor that stabilizes in the nucleus upon exposure to hypoxic conditions (20, 38) and in turn induces the expression of hypoxia-induced genes. HIF-1 causes increased gene expression for glucose transporters, glycolytic enzymes, angiogenic factor, and erythropoietin by targeting the hypoxic response element in the promoter region (33, 36). Higher HIF-1 mRNA levels after training (40, 43) may produce an increased potential for quick activation of HIF-1 with the onset of hypoxic stimuli after training at altitude (17). However, the response of HIF-1 after exercise training is blunted in response to an absolute intensity exercise protocol l(26). Thus, HIF-1 stabilization may be increased by exercise and factors independent of hypoxia. Indeed, interleukin-1beta, insulin like growth factor I and II, insulin, heregulin, epidermal growth factor TNF alpha, angiotensin-2, and nitric oxide have been shown to be capable of inducing HIF-1 (8, 12, 15, 24, 35, 42). The complex nature of HIF-1 activation serves a common endpoint to deliver oxygen and up-regulate the metabolic machinery of the cell.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) gene expression is strongly induced by exercise (1, 23, 32) contributing to mitochondrial biogenesis and metabolic alterations. Recent data from our lab has shown PGC-1α mRNA to increase with acute exercise, but have a blunted response after 11 and 21 days of endurance training (37). PGC-1α expression is also induced by hypoxia (41) and does not appear to be dependant on HIF-1.   The activation of PGC-1α stimulates mitochondrial biogenesis and hence contributes to increased fatty acid fuel oxidation and subsequent exercise performance (25, 41).

The current view of the mitochondria is not one of many mitochondria, but of a single mitochondrial complex within a cell where the solitary shapes of mitochondria are considered to be sections of mitochondrial tubules. Fusion (growth and development) of these tubules has been shown in healthy conditions such as exercise and weight loss (10, 31) while fission (degradation, dysfunction) is associated with obesity and disease (2, 3). We have recently observed an increase in Mitofusin 2 (MFN2) mRNA, a mitochondrial fusion gene with acute exercise throughout 21 days of endurance training (37). It has been suggested that the balance between fusion and fission may play a central role in the metabolic consequences of hypoxia(21).

These two distinct pathways of metabolic alteration with hypoxia (1. HIF-1 and 2. PGC-1α) have very different effects. The HIF-1 pathway leads to increased reliance on carbohydrate substrates and the PGC-1α pathway leads to increased reliance on fat substrates. Clearly, investigations are needed to discern the pathways and applied metabolic outcomes associated with hypoxia. This information will allow for protocol development for not only altitude/hypoxia tolerance, but also for exercise performance, both of which may be critical to mission success in harsh environments.

This investigation aimed to determine metabolic, morphologic, and oxidative stress gene expression related to mitochondrial development after exposure to simulated high altitude (5000 m). The novel use of this very high altitude will allow insight into mitochondrial response using a very robust hypoxic stimulus. These data have implications into the efficacy of training at such a high altitude in order to stimulate enhanced exercise performance and thus increased probability of mission success.

METHODS

Participants
Twelve male participants (25 ± 2 yrs, 178 ± 7 cm, 79 ± 8 kg, 4.2 ± 0.6 L•min^{-1} completed the study. Subjects completed a Physical Activity Readiness Questionnaire (PAR-Q) and were briefed on the experimental protocol and possible risks prior to giving written informed consent. All procedures were approved by the University Institutional Review Board (The University of Montana, Missoula, MT).

Preliminary Testing
Body composition was measured using hydrodensitometry. Underwater mass was measured with a digital scale (Exertech, Dreshbach, MN). Body density was corrected for estimated residual lung volume (3) and converted to percent body fat using the Siri equation (9). A graded maximal exercise test (starting at 95 W, and increasing 35 W every 3 minutes) was completed on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) to determine maximal aerobic capacity (VO_{2max}) and the power output associated with VO_2max (W_{max}). Expired gases were measured during the test, using a calibrated metabolic cart (ParvoMedics, Inc., Salt Lake City, UT). VO_{2max} was assigned to the highest achieved oxygen uptake recorded during the test. W_{max} was calculated by adding the power output in the last completed stage to the fraction of time spent in the uncompleted stage multiplied by 35.

Experimental Protocol
Design.
Participants completed 2 trials by cycling for 90 minutes in laboratory conditions and recovering in 2 different hypoxic environments [975 meters and 5000 meters] using a randomized, counterbalanced cross-over design over the span of 2 weeks, with a minimum of 7 days between trials. All trials were completed in a temperature, humidity, and hypoxia (Colorado Altitude Training, Louisville, CO) controlled environmental chamber (Tescor, Warminster, PA) in ambient conditions of 25°C and 40% relative humidity. Participants kept an exercise record for 2 days before and a dietary record for 24 h before the initial trial and replicated exercise and diet for these periods before the remaining trials. Additionally, participants abstained from exercise 24 hours before each trial. Following an overnight 12 hour fast, participants arrived at the laboratory in the early morning to complete testing. Upon arrival to the laboratory nude body mass was measured (CW-11, Ohaus Corporation, Pine Brook, NJ). Participants performed a 90 minute interval cycling protocol as follows: 1) 10-minute warm up at ~55% VO_{2max}, 2) series of ten intervals, which included two minutes at approximately 80% VO_{2max} followed by four minutes at ~50% VO_{2max}, 3) after the series of 10 intervals, completing 12 minutes at ~60% VO_{2max} followed by 10 minutes at ~50% VO_{2max}. Immediately after the ride, subjects toweled off and changed into dry clothes. Participants remained in a sitting position throughout the 6 hour recovery period. Participants received a liquid carbohydrate beverage immediately prior to entering the chamber (1.2 g•kg^{-1}) and solid feedings (1.28 g•kg^{-1} carbohydrate, 0.15 g•kg^{-1} fat, and 0.29 g•kg^{-1} protein) at 2 and 4 hours into recovery. Nude body mass was measured following the ride and at the end of the 6 hour recovery period. Participants consumed 8 ml•kg^{-1} of water during the ride and ad-libitum water intake during the recovery.

Biopsies. Muscle biopsies were taken before and after exercise, and at the end of the 6 hour recovery period for each trial. Biopsies were taken from the *vastus lateralis* muscle using a 5 mm Bergstrom percutaneous muscle biopsy needle with the aid of suction (2). All subsequent biopsies during a given trial were obtained from the same leg using a separate incision 2 cm proximal to the previous biopsy. After excess blood, connective tissue, and fat were removed, tissue samples were stored in RNA Later or immersed in liquid nitrogen and stored at -80°C for later analysis.

Blood Samples. A 10 mL blood sample was taken from an antecubital vein before and after exercise and at 2, 4, and 6 hours during recovery. Immediately, 2 capillary tubes were filled with 100ul of blood each and placed in a hematocrit centrifuge for 3 minutes, 15,290 x g (A13, Jouan, Winchester, VA) for analysis of hematocrit. The proportion of blood cells to serum was measured in each tube and the average of the two measures was used to determine hematocrit. The remainder of the blood sample was then centrifuged at 7500 x g for 20 minutes at 4°C (MR 22i, Jouan, Winchester, VA). Plasma was removed and stored at -80°C until subsequent analyses.

Respiratory Parameters. Expired gases were measured during recovery at 2:30, 4:30, and 5:55 to evaluate differences in oxygen consumption and respiratory function among trials. Each collection was collected for 5 min, with the last 3 min averaged to represent the sample period.

Pulse Oximetry. Blood oxygen saturation was evaluated before and after exercise, and during recovery at 0:30, 2:30, 4:30, and 6:00 using a pulse oximeter (Nonin Onyx II 9550, Plymouth, MN).

Analysis

Skeletal Muscle RNA isolation. An 8-20 mg piece of skeletal muscle will be homogenized in 800ul of Trizol using an electric homogenizer. The samples are then incubated at room temperature for 5 minutes after which 200ul of chloroform per 1000ul of Trizol is added and shaken vigorously by hand. After an additional incubation at room temperature for 2-3 minutes the samples are centrifuged at 12,000 g for 15 minutes and the aqueous phase was transferred to a fresh tube. The RNA is then precipitated by adding 500ul of isopropyl alcohol pre 1000ul of initial Trizol and incubated overnight at -20°C. The next morning samples are centrifuged at 12,000g for 10 minutes at 4°C and the RNA is washed by removing the supernatant and adding 1000ul of 75% ethanol per 1000ul of initial Trizol. The samples are then mixed by vortex and centrifuged at 7,500g for 5 minutes at 4°C. The RNA is then redissolved in 100 ul RNase-free water after the supernatant is removed and the RNA pellet was dried. The RNA is then cleaned using the RNeasy mini kit (Qiagen) according to the manufactures protocol using the additional DNase digestion step (DNA mini kit, Qiagen). RNA is then quantified using a nano-spectrophotometer.

cDNA synthesis. First-strand cDNA synthesis is achieved using Superscript-fist strand kit (Invitrogen) according to the manufactures protocol. Each sample within a given subject will

contain the same amount of RNA (400 – 1000 ng). The resulting cDNA was then diluted using RNase free water in order to have adequate volume for RT-PCR and frozen for later RT-PCR analysis.

Real time RT-PCR. Each 25ul reaction volume will contain 500nM primers, 250nM probe (PrimeTime qPCR assay, Integrated DNA technologies), 1x FastStart TaqMan Probe master (Roche Applied Science), and 2.5 ul of sample cDNA. PCR will then run using the Bio-Rad I Cycler iQ5 Real-Time PCR Detection system (Bio-Rad) using a 2-step Roche protocol.

Oxidative Stress. Plasma aliquots were assayed for an oxidative stress biomarker panel used previously by our group (6,8). Two biomarkers, plasma trolox-equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP), were chosen to evaluate blood plasma antioxidant capacity. Oxidative damage was evaluated in plasma by determination of protein carbonyls and lipid hydroperoxides content.

Antioxidant Capacity. Plasma antioxidant capacity was measured by the plasma trolox-equivalent antioxidant capacity technique whereby a radical cation of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) reaction is quenched by sample-specific antioxidant fortifications (5). Total plasma antioxidant potential was determined by the ferric reducing antioxidant potential assay according to the methodology of (1). Both TEAC and FRAP assays result in quantifiable colorimetric solutions which are visualized spectrophotometrically. Lipid peroxidation was determined by the ferrous oxidation-xylenol orange assay (7). In brief, ferrous ions are oxidized by lipid hydroperoxides to ferric ions and subsequently react with the ferrous sensitive dye containing xylenol orange. In the presence of lipid hydroperoxides, this reaction forms a spectrophotometrically quantifiable complex. Protein carbonyls were analyzed using a commercially available ELISA kit (Zentech Technology, Dunedin, New Zealand). All assays were performed in triplicate and exhibited within sample coefficients of variation between 2% and 5%. Prior to analysis, all plasma samples were assayed in quadruplicate for protein concentration based on the methods of Bradford et al, (1976) and adjusted to 4 mg/ml protein using a phosphate buffer (4). All oxidative stress biomarkers were normalized for plasma volume shifts experienced during the three trials.

Statistics

Muscle glycogen, substrate utilization, mRNA of metabolic genes and oxidative stress markers were analyzed using a repeated measure ANOVA (trial*time). A probability of type I error less than 5% was considered significant ($p < 0.05$). All data is reported as means ± SE.

Methodology References:

1. Benzie, IF, Strain, JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239: 70-76, 1996.

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5. Cao, G, Russell, RM, Lischner, N, Prior, RL. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J Nutr* 128: 2383-2390, 1998.

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7. Nourooz-Zadeh, J, Tajaddini-Sarmadi, J, Wolff, SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* 220: 403-409., 1994.

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RESULTS:

Gene Expression:
Gene expression for mitochondrial biogenesis related genes was reduced with hypoxia (simulated, 5000 m elevation) in 8 of 12 genes ($p < 0.05$, figures 1-4) as compared to normoxia (975 m elevation). The other 4 genes were reduced, but not to a significant level ($p > 0.05$).
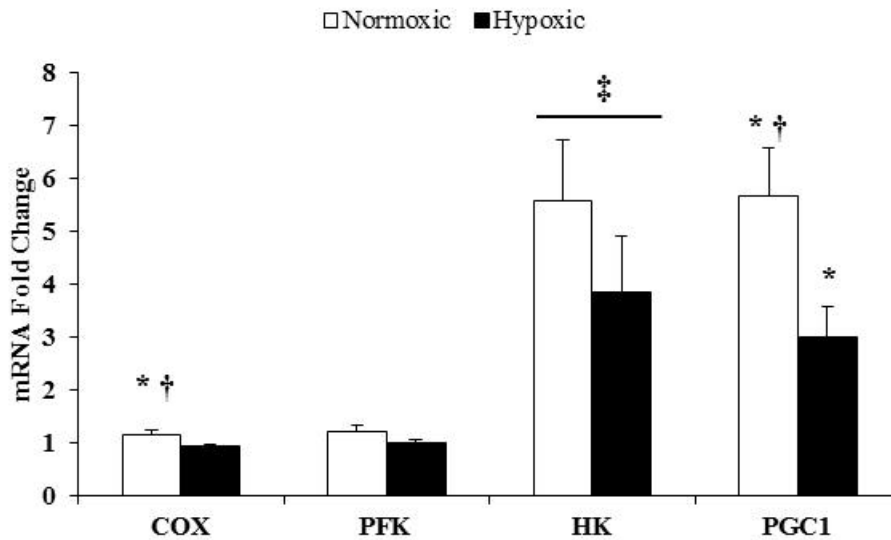
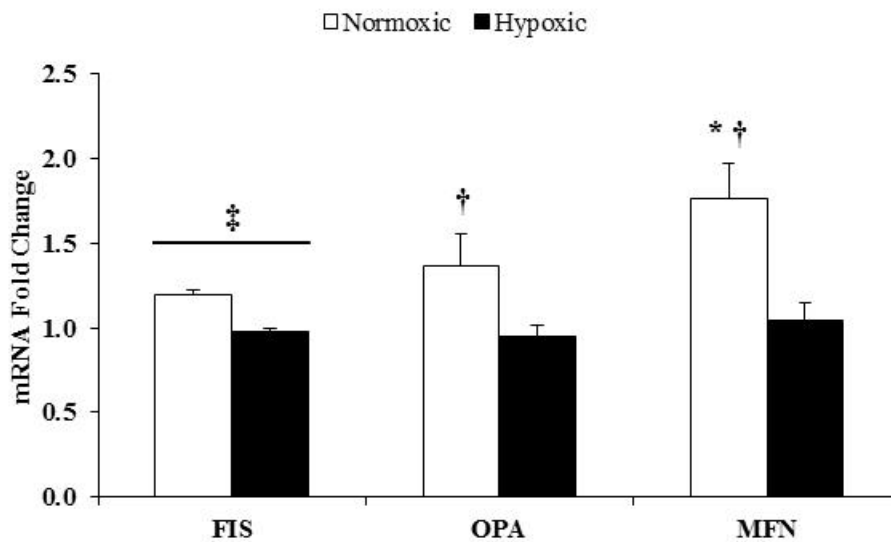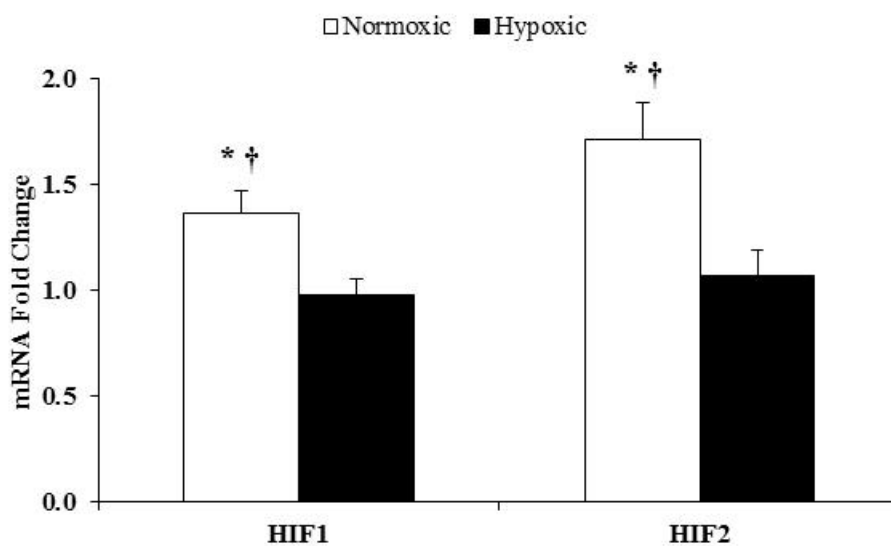**Figure 1.** Metabolic gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$ from the hypoxic trial, ‡ $p < 0.05$ from pre (main effect of trial). COX, cytochrome c oxidase subunit 4; PFK, phoshofructokinase; HK, hexokinase; PGC1, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.
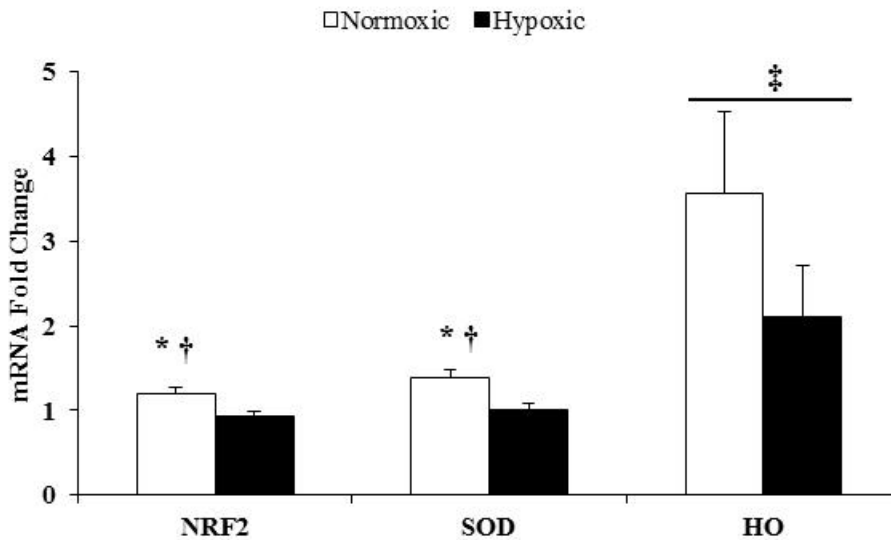
Figure 2. Oxidative stress related gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$

from the hypoxic trial, ‡ p < 0.05 from pre (main effect of trial).   NRF2, nuclear factor (erythroid-derived 2)-like 2 also known as NFE2L2; SOD, superoxide dismutase 2; HO, heme oxygenase 1.


Figure 3.   Mitochondrial morphology related gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * p < 0.05 from pre; † p < 0.05 from the hypoxic trial, ‡ p < 0.05 from pre (main effect of trial).   FIS, mitochondrial fission protein 1; OPA, optic atrophy 1; MFN, mitofusin 2.

Figure 4.   Hypoxia regulation gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * p < 0.05 from pre; † p < 0.05 from the hypoxic trial. HIF1, hypoxia inducible factor 1 alpha subunit; HIF2, hypoxia inducible factor 2 alpha subunit.

Oxidative stress:

Trolox equivalent antioxidant capacity of plasma (TEAC): Time (p=0.044, Pre vs Post, 1Hr; 6Hr vs 1Hr, 2Hr) and trial (p=0.021) main effects were present. Significant trial differences were present between Normoxic and Hypoxic recovery at 1Hr, 2Hr, and 6Hr recovery periods. Based on the fact that plasma TEAC is heavily influenced by plasma urate concentrations, this finding likely reflects increased purine metabolism during exercise. The metabolic pathway responsible for urate production, reflected by increased TEAC values, results in the production of reactive oxygen species during two enzymatically catalyzed reactions. Given the similarities to previous findings with similar study designs, we interpret this finding to indicate that exercise elicited an increase in oxidative stress during recovery, while hypoxic recovery attenuated this response.
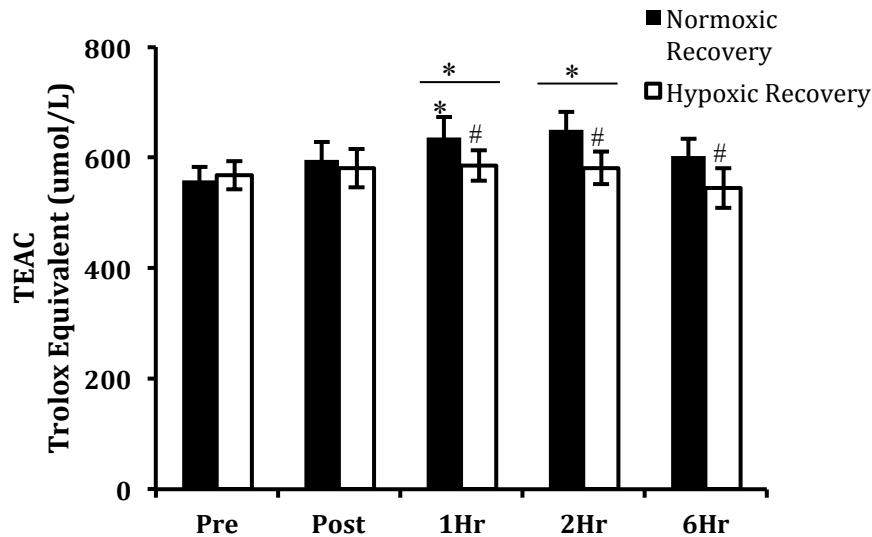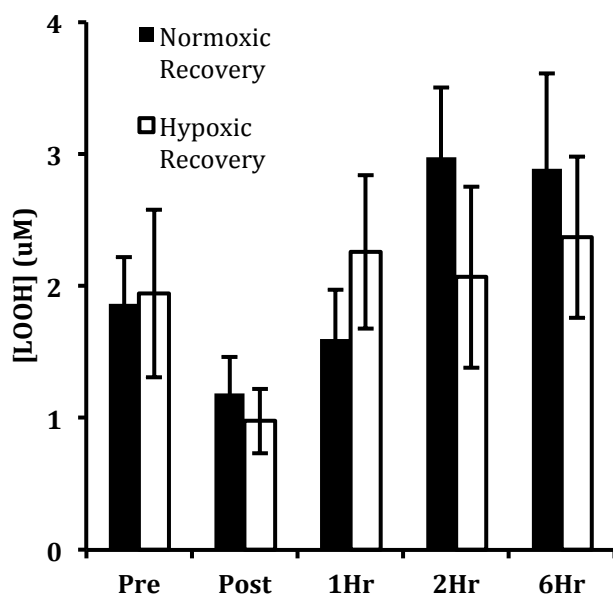

Figure 5. TEAC values pre, post, and under hypoxic recovery stress. Data are means +/- SEM; * denotes significantly different from Pre, # denotes significantly different from the Normoxic Recovery condition.

Ferric reducing ability of plasma (FRAP): Time (p=0.031, Post vs 6Hr) main effects and interaction effects (p=0.030) were present. Pre-Post differences approached significance (p=0.069). Significant trial differences were present between Normoxic and Hypoxic recovery at 6Hr recovery period with differences approaching significance 1Hr following exercise (p=0.072). Plasma FRAP values at the 6Hr time point were significantly lower than Pre during Hypoxic Recovery (p=0.020). These findings, while less conclusive than TEAC, generally support TEAC results suggesting that redox responses to Hypoxic Recovery were altered as compared to the Normoxic Recovery trial. In support, analysis of the percent change in plasma FRAP (Time main effect p=0.032; Trial main effect p=0.066) was observed including a significant drop in the percent change in at the 6Hr recovery time point (p=0.045) from Hypoxic Recovery samples.
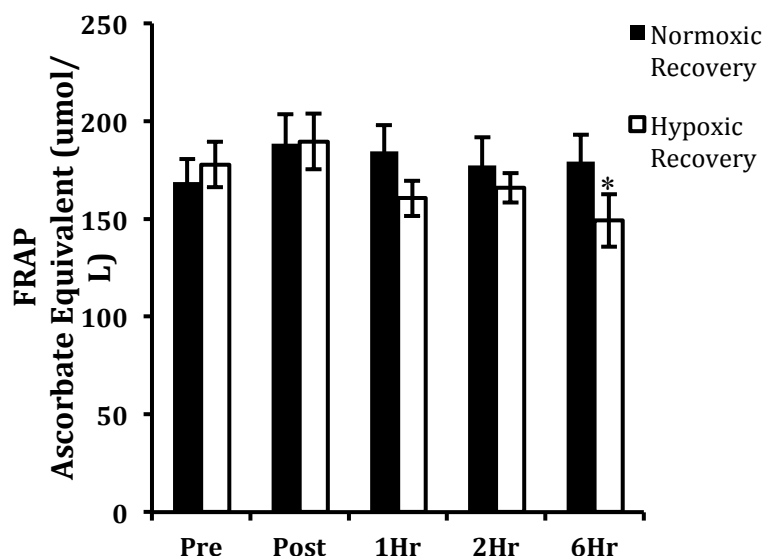
Figure 6. FRAP values pre, post, and under hypoxic recovery stress. Data are means +/- SEM; * denotes significantly different from Pre.

Lipid hydroperoxides (LOOH): Time main effects (p=0.041, Post vs all other time points) were observed for plasma LOOH. Sample variances were unusually large for this investigation and cannot be explained currently. While numeric differences between means suggest elevations in plasma LOOH following Normoxic Recovery vs Hypoxic Recovery, this conclusion was not supported statistically.

Figure 7. LOOH concentrations pre, post, and under hypoxic recovery stress. Data are means +/- SEM.

Protein carbonyls (PC): Time main effects (p=0.044, Post vs all other recovery time points) were observed for plasma PC. The percent change in plasma PC support findings with

absolute values in that 1Hr (p=0.005) were elevated as compared to Post (Post-2Hr, p=0.069). The percent increase over Pre values was most notable at 1Hr into the Normoxic Recovery (p=0.004) where an average increase of 16% was observed. As with plasma LOOH, examination of significant differences between trials is not warranted statistically, however, examination of post hoc analyses reveals an increase in 1Hr post Hypoxic Recovery as compared to the immediately post value.
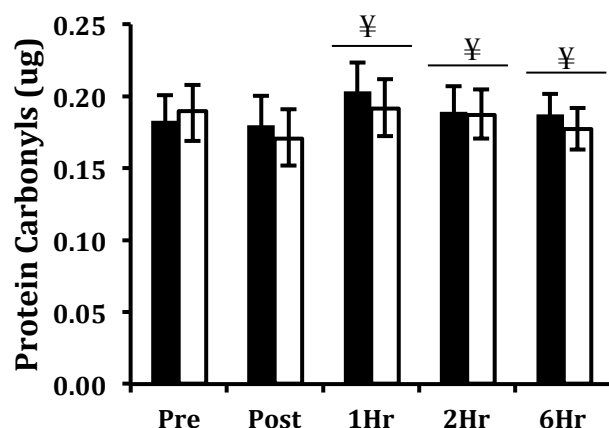
Figure 8. Protein Carbonyls pre, post, and under hypoxic recovery stress. Data are means +/- SEM; ¥ denotes significantly different from Post.

Interleukin-6 (IL-6): No main effects (time, p=0.484; trial, p=0.327) were not present for plasma IL-6 changes. Note that values required a polynomial regression. Notably, however, these IL-6 values were plagued by several subjects for which a consistent order of magnitude difference was noted for all trials and time points. As such we performed the percentage increase analyses here too and observed a significant trial difference (p=0.013) in that the post exercise increase in plasma IL-6 was significantly higher during the Hypoxic Recovery as compared to the Normoxic Recovery.

Summary:

Gene Expression:

The novel outcome of this investigation was the reduced gene expression of mitochondrial related genes with a very robust hypoxic intervention. Initially, this would appear to be contradictory to previous research that has shown increased mitochondrial biogenesis with altitude/hypoxic exposure (4, 6, 10, 14, 29, 30, 39, 40). However, these investigations did not investigate the effects of an aggressive hypoxic stimulus. At higher altitudes mitochondrial related function has been shown to decline (7, 9, 18, 19, 22, 27, 28).

From this, it may be speculated that a mild hypoxic stress may produce beneficial mitochondrial adaptations. In contrast, exposure to more extreme hypoxia may diminish the degree of mitochondrial adaptation. This speculation may explain the ambiguity of previous research on metabolic adaptations with altitude.

Future research should investigate a dose response relationship between the degree of hypoxia and the gene expression of mitochondrial associated markers. We hypothesize that there may be a critical altitude at which enhanced mitochondrial adaptation can occur after which diminished response to the magnitude of inhibition of response occurs.

Oxidative Stress:

The collective effect of these data strongly suggest that the exercise stimulus elicitied an oxidative stress response that became evident during the recovery period. The interesting discovery is that Hypoxic Recovery appeard to blunt many of these findings as compared to the Normoxic Recovery.

Examination of the data do raise questions about why some of the typical post exercise findings were not observed in the current samples. However, some of the expected common post exercise increases in these markers were not statistically significant dispite trends. Some of these findings may related to to hydration status of the subjects during the recovery period.

The decreased oxidative stress that we observed may provide the mechanism by which mitochondrial stimuli is reduced. Oxidative stress may be stimuli for mitochondrial adaptation that does not continue to increase in a linear manner at very high levels of hypoxia.

Findings from year 3 – Laboratory Study Series

INTRODUCTION

It has been largely assumed since 1962 that hypoxic conditions and/or altitude stimulate muscle oxidative capacity. This notion comes from the observation that active Peruvian miners had 78% more cytochrome c reductase and 16% more myoglobin than low land controls (34). Five years later it was noted that endurance exercise was a potent stimulator of mitochondrial enzymes (16). The data from these two studies formed the consensus that muscle hypoxia (from exercise or environment) was an important stimulus for mitochondrial development. In the early 1990's this tenant was challenged by data before and after mountaineer expeditions to the Himalayas. The findings of these studies demonstrated a loss of muscle cross sectional area, decreased mitochondrial volume, and decreased maximal aerobic capacity (9, 18, 19). Additionally, after return from these expeditions lipofuscin, a mitochondrial breakdown by-product, was increased (28). These findings of reduced oxidative capacity were further confirmed by "Expedition Everest II" (27). Low levels of mitochondrial capacity have also been shown in the high altitude residents of La Paz (7) and in Sherpa's (22). Paradoxically, these high land populations have excellent physical performance at altitude.

When exercise training under hypoxic conditions, but recovering in a normoxic environment, a slightly different story emerges (train high, live low). When this type of short-term exposure is incorporated into a training paradigm mitochondrial density, maximum aerobic capacity, citrate synthase activity, and anaerobic performance is enhanced over normoxic control exercise (4, 6, 11, 14, 29, 30, 39, 40). Functionally, increased glucose metabolism shown with altitude exposure and acclimatization (5) may be more efficient in that this adaptation would lead to more ATP per molecule of oxygen. The paradox with short-term and long-term metabolic adaptations is yet to be completely understood, however it may be related to the recovery process and specifically indications of decreased protein synthesis during hypoxia (13).

The mechanisms to which hypoxic adaptations occur are even less clear, but seem to be regulated by hypoxia-inducible factor 1 (HIF-1) (36). HIF-1 is a transcription factor that stabilizes in the nucleus upon exposure to hypoxic conditions (20, 38) and in turn induces the expression of hypoxia-induced genes. HIF-1 causes increased gene expression for glucose transporters, glycolytic enzymes, angiogenic factor, and erythropoietin by targeting the hypoxic response element in the promoter region (33, 36). Higher HIF-1 mRNA levels after training (40, 43) may produce an increased potential for quick activation of HIF-1 with the onset of hypoxic stimuli after training at altitude (17). However, the response of HIF-1 after exercise training is blunted in response to an absolute intensity exercise protocol l(26). Thus, HIF-1 stabilization may be increased by exercise and factors independent of hypoxia. Indeed, interleukin-1beta, insulin like growth factor I and II, insulin, heregulin, epidermal growth factor TNF alpha, angiotensin-2, and nitric oxide have been shown to be capable of inducing HIF-1 (8, 12, 15, 24, 35, 42). The complex nature of HIF-1 activation serves a common endpoint to deliver oxygen and up-regulate the metabolic machinery of the cell.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) gene expression is strongly induced by exercise (1, 23, 32) contributing to mitochondrial biogenesis and metabolic alterations. Recent data from our lab has shown PGC-1α mRNA to increase with acute exercise, but have a blunted response after 11 and 21 days of endurance training (37). PGC-1α expression is also induced by hypoxia (41) and does not appear to be dependant on HIF-1.   The activation of PGC-1α stimulates mitochondrial biogenesis and hence contributes to increased fatty acid fuel oxidation and subsequent exercise performance (25, 41).

The current view of the mitochondria is not one of many mitochondria, but of a single mitochondrial complex within a cell where the solitary shapes of mitochondria are considered to be sections of mitochondrial tubules. Fusion (growth and development) of these tubules has been shown in healthy conditions such as exercise and weight loss (10, 31) while fission (degradation, dysfunction) is associated with obesity and disease (2, 3). We have recently observed an increase in Mitofusin 2 (MFN2) mRNA, a mitochondrial fusion gene with acute exercise throughout 21 days of endurance training (37). It has been suggested that the balance between fusion and fission may play a central role in the metabolic consequences of hypoxia(21).

These two distinct pathways of metabolic alteration with hypoxia (1. HIF-1 and 2. PGC-1α) have very different effects. The HIF-1 pathway leads to increased reliance on carbohydrate substrates and the PGC-1α pathway leads to increased reliance on fat substrates. Clearly, investigations are needed to discern the pathways and applied metabolic outcomes associated with hypoxia. This information will allow for protocol development for not only altitude/hypoxia tolerance, but also for exercise performance, both of which may be critical to mission success in harsh environments.

This investigation series has aimed to determine metabolic, morphologic, and oxidative stress gene expression related to mitochondrial development after exposure to simulated altitudes (0-5000 m). These data have implications into the efficacy of training at such a high altitude in order to stimulate enhanced exercise performance and thus increased probability of mission success.

METHODS

Participants
Active males between the ages of 18-40(n=12) were recruited from the University of Montana and surrounding area to examine the effects of altitude exposure and exercise elicit blood oxidative stress at 4 stimulated altitudes on blood oxidative stress markers. Each participant also completed a physical activity readiness questionnaire (PAR-Q) to determine their physical activity readiness. Written informed consent approved by the University of Montana IRB was given to each participant prior to the commencement of data collection.

Baseline Testing
A measure of percent body fat was obtained by using hydrodensitometry method. The recorded weights were corrected for residual lung volume (residual lung volume=

(0.0115*Age) + (0.019*Height)-2.2.4). The underwater weights were obtained using a digital scale (Exertech, Dresbach, MN). Participants repeated trails until 3 hydrostatic weight values within 100 g were obtained. The relationship between hydrostatic weight and weight in the air were used to calculate body volume and converted to the percent fat using the Siri Equation (BF= $(4.95/\rho-4.59)*100$).

Participants were given a maximal cycle ergometer test to measure their aerobic fitness. The test was performed on an electronically braked cycle ergometer (Velotron, Racermater Inc., Seattle, WA). Each participant completed a hypoxic (3000m) and normoxic (975m) graded exercise test on the electronically braked bike. The initial workload of 95 W was incrementally increased every 3 minutes by 35 W until subjects achieved volitional fatigue. Gas expiration was collected during exercise and analyzed in 15 second intervals. Subsequent steady stat workloads were determined by the power output associated with VO_2peak values (Wmax) from both altitudes.

Steady State Exercise Trials
Participants completed three 1-hour steady state exercise sessions on a cycle ergometer (Velotron, Racermate Inc., Seattle, WA) in a randomized counter-balanced cross-over design (Figure 1). Participants were asked to abstain from physical exercise before each trial commenced. Additionally, participants were asked to complete a 12-hour overnight fast. To ensure compliance the participants were asked to complete a 2-day exercise log and a 1-day dietary record, which were replicated prior to all steady state exercise sessions. Pulse oximetry was monitored pre, after cycling for 45 minutes, post exercise, and every hour during the 6-hour recovery using pulse oximeter (Nonin Onyx Finger Pulse Oximeter, Nonin Medical Inc, Plymouth, USA). Participants consumed 600 mL of water during each interval exercise and 600 mL during the post-exercise recovery. Initial interval trials were performed in normoxic conditions at 975 meters, the altitude of the laboratory. Upon cessation of each exercise bout the participants recovered for 6 hours at a randomized simulated altitude of 0 meters, 1667 meters, 3333 meters, or 5000 meters. The environmental chamber was set at 40 % relative humidity.
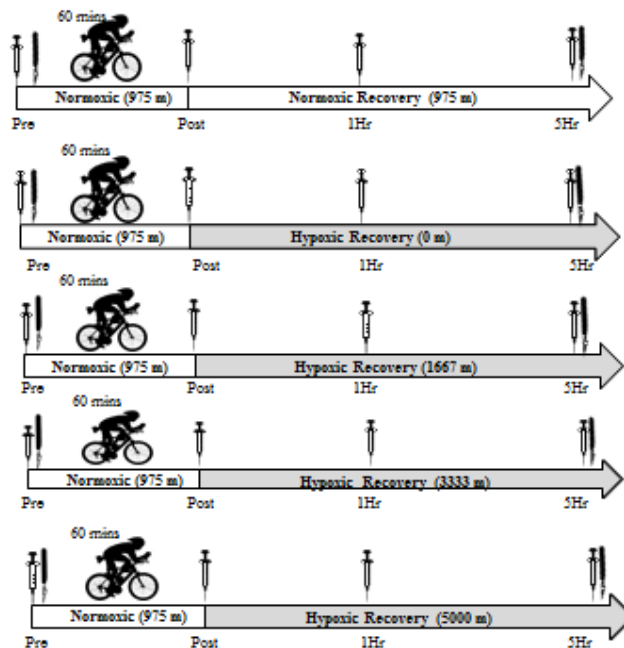
Figure 1. Study Design. Participants performed in identical 60-minute interval cycle ergometer exercise session at normoxic conditions (975 m altitude) indicated by an *open arrow*. In a randomized counter-balanced cross-over design, participants recovered for 6 hrs at 0 m, 1667 m, 3333 m, and 5000m (normobaric hypoxia chamber) indicated by a *shaded arrow*. Blood samples were obtained pre- (PRE), post-(POST),1 hr post-(1HR), and 5 hrs (5HR) post-exercise.

Experimental Blood Samples
There were a total of 16 whole blood samples (4 per trial) collected using a venipuncture technique. The blood draws occurred pre- (Pre), post –(Post), 1- (1Hr), 5(Hr) hours post exercise from the antecubital vein with sodium heparinized vacutainers. Blood samples were centrifuged at 1000 x g for 15 minutes at 4 ° C , aliquot and immediately stored at -80 °C until subsequent biochemical analysis of blood plasma oxidative damage and antioxidant activity biomarkers.

Muscle Biopsies and Tissue Storage
There were a total of 8 (2 per trial x4 trials) skeletal muscle biopsies (4 from each leg). The samples were taken from the vastus lateralis muscle using a percutaneous needle biopsy procedure. The area was treated with local anesthesia (1 % lidocaine) through a subcutaneous and intramuscularly prior to incision. Following local anesthesia a small incision (approximately 0.25 inches) was made and approximately 50-100 mg of tissue was obtained. Incisions were closed through a single suture, supported with Steri-Strip, and covered with sterile adhesive bandage. Muscle tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Biochemical Assays – Gene Expression

An 8–20 mg piece of skeletal muscle was homogenized in 800 µl of Trizol (Invitrogen, Carlsbad CA, Cat# 15596-018) using an electric homogenizer (Tissue Tearor, Biosped Products Inc., Bartlesville OK). Samples were then incubated at room temperature for 5 min after which 200 µl of chloroform per 1000 µl of Trizol was added and shaken vigorously by hand. After an additional incubation at room temperature for 2–3 min the samples were centrifuged at 12,000 g for 15 min and the aqueous phase was transferred to a fresh tube. mRNA was precipitated by adding 400 µl of isopropyl alcohol and incubated overnight at -20 °C. The next morning samples were centrifuged at 12,000 g for 10 min at 4 °C and the mRNA was washed by removing the supernatant and adding 800 µl of 75% ethanol. Samples were vortexed then centrifuged at 7500 g for 5 min at 4 °C. mRNA was re-dissolved in 100 µl RNase-free water after the supernatant was removed and the mRNA pellet was dried. The RNA was further purified using the RNeasy mini kit (Qiagen, Valencia CA, Cat# 74104) according to the manufacturer's protocol using the additional DNase digestion step (RNase-free DNase set, Qiagen, Valencia CA). RNA was quantified using a nano-spectrophotometer (nano-drop ND-1000, Wilmington DE), and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). First-strand cDNA synthesis was achieved using Superscript-first-strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. Each sample within a given subject was adjusted to contain the same amount of RNA. The resulting cDNA was then diluted 2× using RNase free water in order to have ample volume for RT-PCR and frozen for later analysis. For Real time RT-PCR, each 25 µl reaction volume contained 500 nM primers, 250 nM probe (PimeTime qPCR assay, Integrated DNA technologies), 1 × Brilliant III Ultra Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA), and 2.5 µl of sample cDNA. PCR was then run using the Agilent Stratagene Mx3005P Real-Time PCR detection system (Agilent Technologies, Santa Clara, CA) using a 2-step Roche protocol (1 cycle at 95 °C for 3 min, followed by 45 cycles of 95 °C for 5 s followed by 60 °C for 20 s. Five housekeeping genes were analyzed (β-actin, cyclophillin, and GAPDH, ribosomal protein S18, β-2-myoglobin) using qbase+ geNorm software (Biogazelle, Ghent, Belgium). geNorm analysis found β-actin and cyclophillin to be the two most stable housekeeping genes (Hellmans et al. 2007, Vandesompele et al. 2002), and all genes were normalized to the geometric mean of β-actin and cyclophillin. Quantification of mRNA for genes of interest was calculated on pre and 3 h post muscle samples using the $2^{-\Delta\Delta CT}$ method corrected for PCR efficiency (Pfaffl, 2001).

Biochemical Assays – Oxidative Stress

Panels of biochemical assays were performed to quantify and characterize blood oxidative stress during exercise and subsequent post-exercise recovery. To measure antioxidant capacity, the ferric reducing ability of plasma (FRAP) was performed. FRAP assay utilizes a colormetric reaction of ferric to ferrous tripyridyltriazine (TPTZ) reduction by plasma antioxidants at an acidic pH. The reduction of TPTZ is proportional to blood plasma antioxidant capacity and was quantified by absorbance spectroscopy at 593 nm. Trolox equivalent antioxidant capacity (TEAC) assay was performed to assess the non-enzymatic antioxidant capacity of the blood plasma. Present antioxidants are scavenge 2,2' azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical anions (ABTS⁻), thus quenching a quantifiable colormetric reaction. Calculated TEAC values for each sample were based on standard reactions with the water-soluble vitamin E analogue Trolox. Uric Acid (UA) is a major

metabolite found in purine substances. UA assay makes use of the catalytic activity of peroxidase the generated H_2O_2. Measurements of H_2O_2 were determined by peroxidase catalyzed oxidation of chromogenic and fluorigenic substrates or by catalyse mediated conversion of alcohols to aldehydes, which were then measured spectrophotometrically using 3-methyl-benzothiazoline-2-one hydrazone (MBTH) and 3-dimethylaminobenzoic acid (DMAB).

To quantify the oxidative damage resulting from the trials and conditions, Nitrotryosine (3NT), protein carbonyls (PC), and lipid hydroperoxides (LOOH) were measured. For the 3NT assay, plasma samples were analyzed via chemiluminescence using a commercial ELISA (EMD Millipore Corporation, Billerica, MA, USA). Plasma samples were prepared according to the manufacturer's directions. For PC, plasma sample protein concentrations were analyzed via absorbance spectroscopy. Plasma samples were diluted to 4 mg/ml accordingly. Protein Carbonyls values were determined by a commercial ELISA (Biocell Corporation Ltd, Papatoetoe, NZ) according to the manufacturer's directions. Ferrous oxidation-xyenol orange assay was used to determine lipid hydroperoxides. The oxidized ferrous ions react with the ferrous sensitive dye contained in the xyenol orange forming a complex that can be quantified through absorbance spectroscopy at a wavelength of 595 nm. Calculated adjustments were made to the blood plasma variables for pre and post exercise plasma volume shifts.

Statistical Analysis
Differences in dependent variables throughout the time points and among the two trials were analyzed using two-way repeated-measures ANOVAs (time × trial). All ANOVAs were performed using SPSS for windows version 19 (Chicago, IL). In the event of a significant f-ratio, Fishers protected least significant difference procedure was used to determine where differences occurred. A probability of type I error less than 5% was considered significant ($p < 0.05$). All data are reported as mean ± SD.

RESULTS:
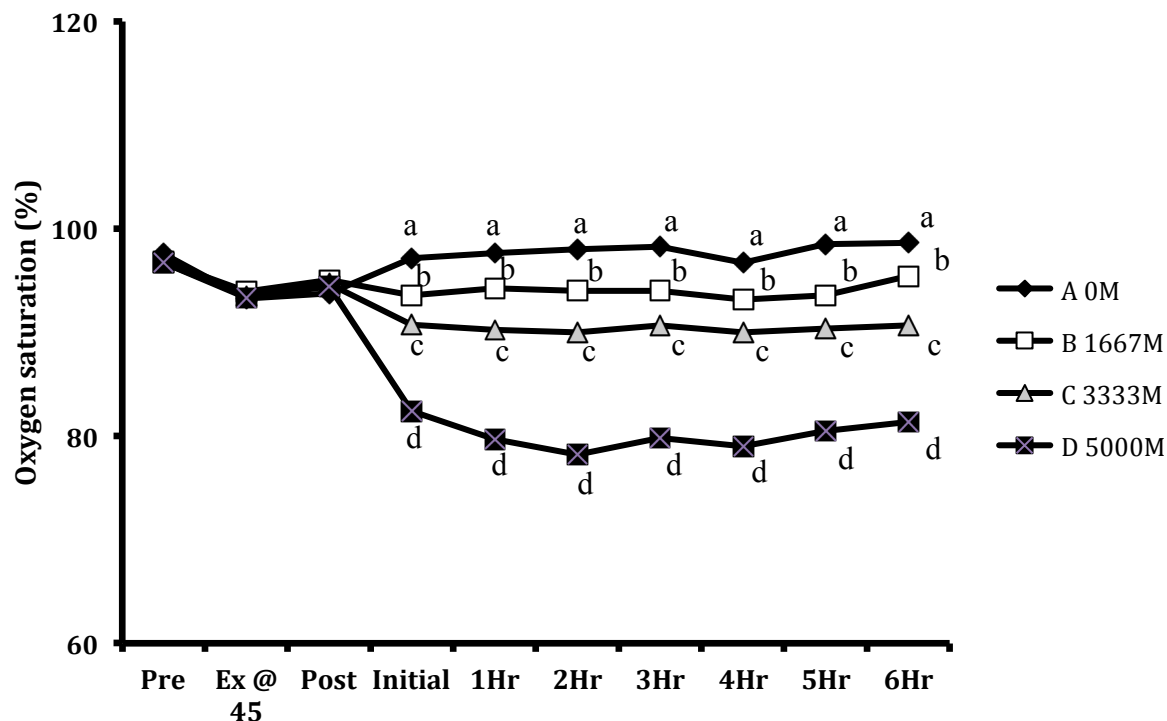
Figure 2. Data are percent oxygen saturation mean ± SE. Finger pulse oximetry at respective time points were used to measure oxygen saturation; *black triangles* represent 0 M recovery*, open squares* represent 1667 M, *shaded triangles* are representative of 3333 M and *black squares* represent 5000 M above sea level. Data reveal statistically similar results for all trials, while there were trial dependent differences in blood oxygen saturation (p<0.05).

Quality Control Measures
RNA 260:280 ratio was 2.05 ± 0.02, while RNA 260:230 ratio was 1.44 ± 0.10. RIN was 8.3 ± 0.1. PCR efficiency was 95.7 ± 1.5%. geNorm analysis revealed high reference target stability, with geNorm M values of 0.416 for CYC and 0.404 for ACTB.
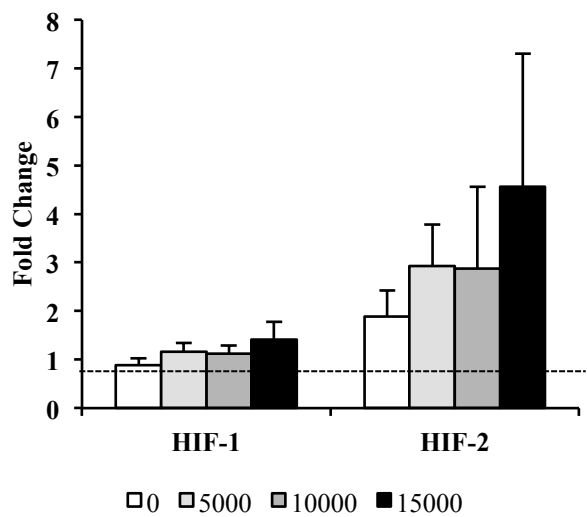
Gene Expression
PCG-1α expression increased with exercise regardless of trial (p = 0.048), but was not different between trials (p = 0.431, Figure 3). Expression of HIF-1 was not different pre- to post-exercise or between trials (p = 0.110 and p = 0.534, respectively). There was a tendency for HIF-2 expression to increase with exercise, although this did not reach statistical significance (p = 0.089). There were no differences in HIF-2 expression between trials (p = 0.507, Figure 4). Expression of HK was not different between trials (p = 0.351), but increased with exercise (p = 0.041). There were no differences in PFK expression with exercise (p = 0.264) or between trials (p = 0.469, Figure 5). There were no differences in expression of FIS, MFN, or OPA with exercise (p = 0.810, p = 0.119, and p = 0.193, respectively) or between trials (p = 0.249, p = 0.671, and p = 0.512, respectively, Figure 6). SOD expression increased with exercise (p = 0.26) but was not different between trials (p = 0.514). There were no

differences in expression of HO and NF with exercise ($p = 0.228$ and $p = 0.853$, respectively) or between trials ($p = 0.291$ and $p = 0.538$, respectively, Figure 7).
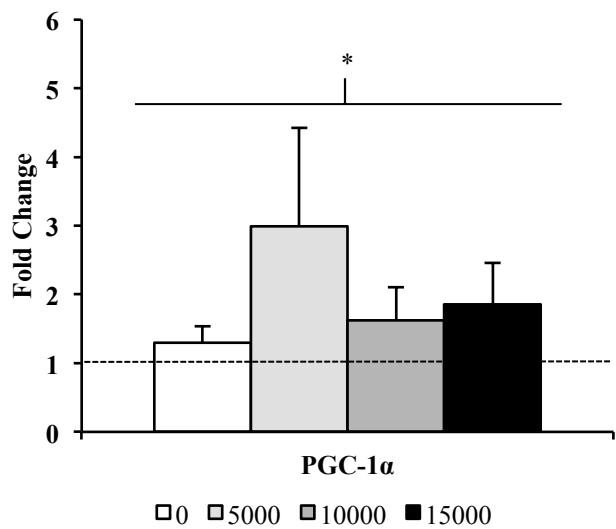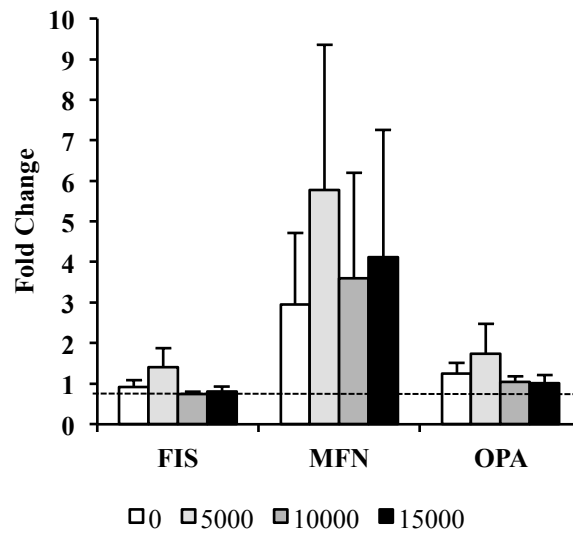

Figure 3.   Relative expression of PGC-1α following exercise at simulated altitudes of 0, 1667, 3333, and 5000 meters. * denotes $p < 0.05$ from pre-exercise.
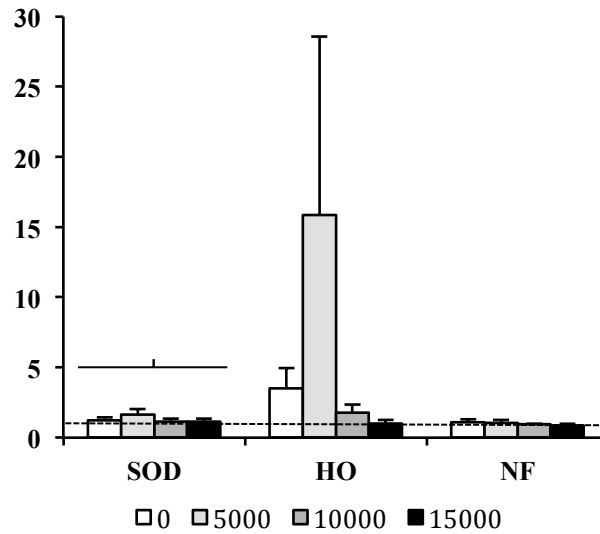

Figure 4.   Relative expression of HIF-1 and HIF-2 following exercise at simulated altitudes of 0, 1667, 3333, and 5000 meters.
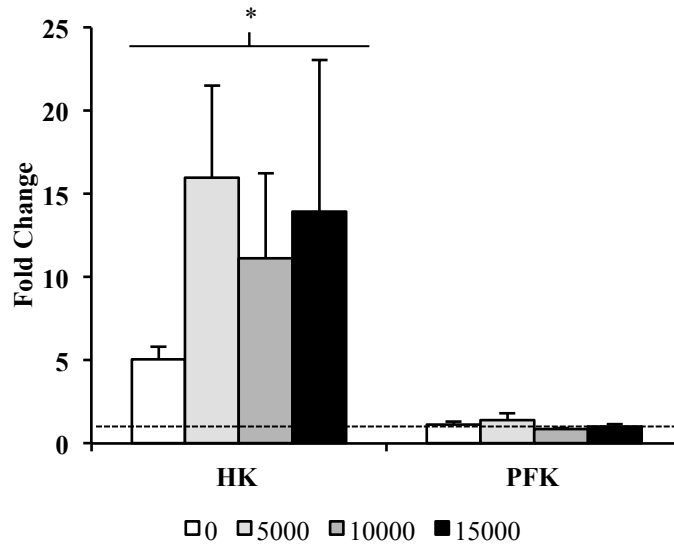
Figure 5. Relative expression of HK and PFK following exercise at simulated altitudes of 0, 1667, 3333, and 5000 meters. * denotes p < 0.05 from pre-exercise.

Figure 6. Relative expression of FIS, MFN, and OPA following exercise at simulated altitudes of 0, 1667, 3333, and 5000 meters.

Figure 7. Relative expression of SOD, HO, and NF following exercise at simulated altitudes of 0, 1667, 3333, and 5000 meters. * denotes $p < 0.05$ from pre-exercise.
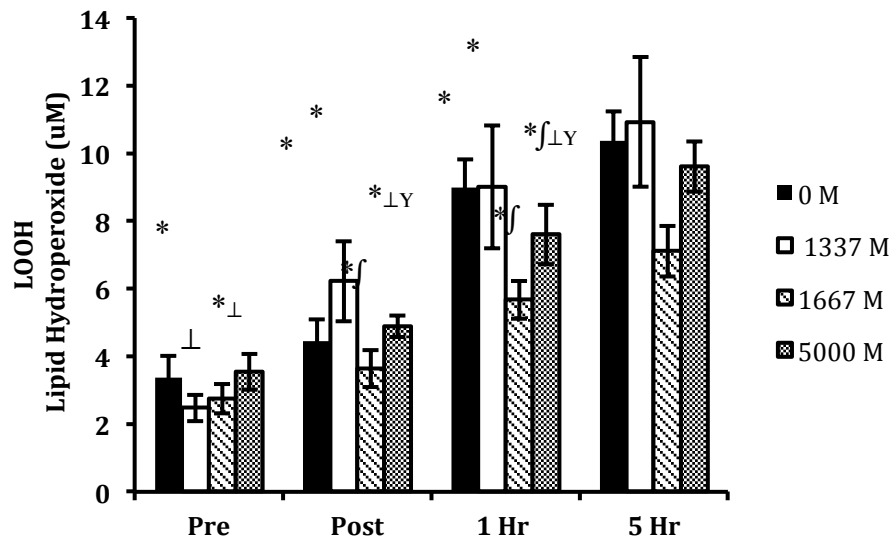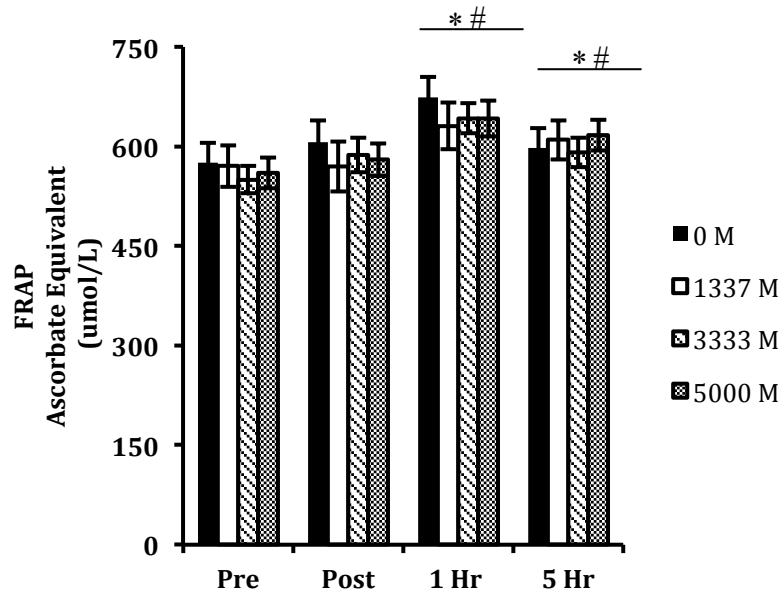

Figure 8. LOOH means are expressed in standard comparison to Lipid hydroperioxide equivalents (µM). *solid black lines* represent 0 M recovery, *open bars* 1667 M, *open stripped bars* are representative of 3333 M and *shaded bars* represent 5000 M above sea level; * significantly different from Pre; # significantly different from normoxic recovery; ∫ significantly different from 0M; ⊥ significantly different from 1337M; Y significantly different from 1667M.

A
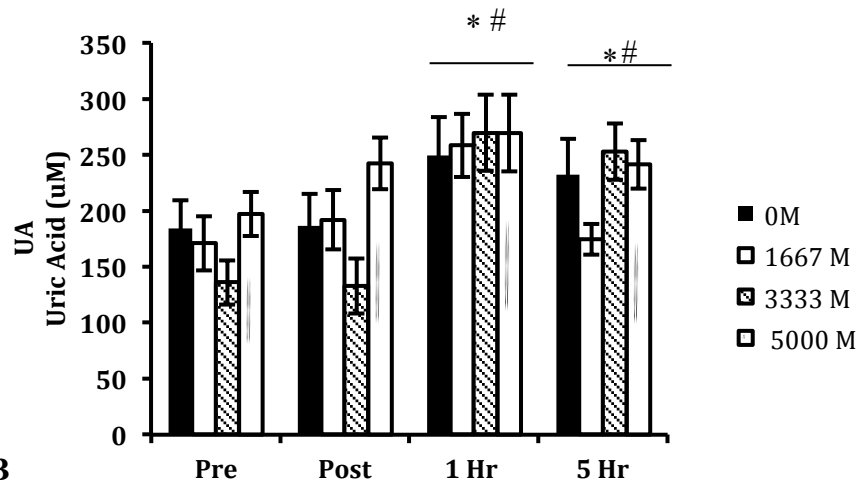
B

C
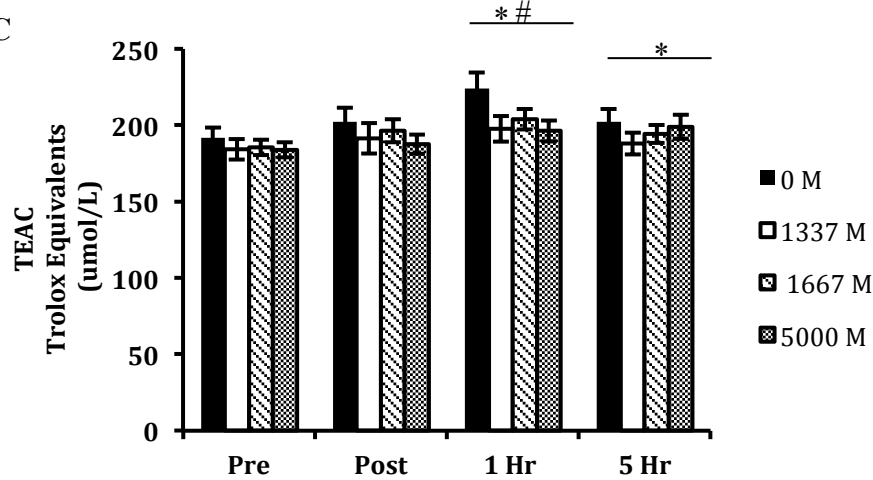

Figure 9.   Data are mean ± SE. **a** UA means are expressed in standard comparison to Uric Acid equivalents (µM). **b** TEAC means are expressed in standard comparison to TROLOX equivalents (µmol/L) **c** FRAP means are expressed in

a standard comparison to Ascorbate in equivalents (µmol/L); *solid black lines* represent 0 M recovery, open *bars* 1667 M, open *stripped bars* are representative of 3333 M and *shaded bars* represent 5000 M above sea level; [*]significantly different from Pre; [#] significantly different from Post.

Summary:

Gene Expression:
The novel outcome of this investigation was the reduced gene expression of mitochondrial related genes with a very robust hypoxic intervention. Initially, this would appear to be contradictory to previous research that has shown increased mitochondrial biogenesis with altitude/hypoxic exposure (4, 6, 10, 14, 29, 30, 39, 40). However, these investigations did not investigate the effects of an aggressive hypoxic stimulus. At higher altitudes mitochondrial related function has been shown to decline (7, 9, 18, 19, 22, 27, 28).

From this, it may be speculated that a mild hypoxic stress may produce beneficial mitochondrial adaptations. In contrast, exposure to more extreme hypoxia may diminish the degree of mitochondrial adaptation. This speculation may explain the ambiguity of previous research on metabolic adaptations with altitude.

We hypothesize that there may be a critical altitude at which enhanced mitochondrial adaptation can occur after which diminished response to the magnitude of inhibition of response occurs. However, our recent findings cannot confirm this hypothesis. When examining the hypoxic dose response relationship we found no differences in gene response between various altitudes. When this data is taken as a whole with our previous data, there appears to be no benefit to the muscle adaptive response when training in hypoxic conditions. Additionally, training in an aggressive hypoxic environment may negatively impact the muscle adaptive response (as noted in study 2, but not confirmed in study 3). The exact mechanisms associated with the paradox of increased mitochondrial function with repeated acute hypoxic exposure and decreased mitochondrial function with long term exposure remain unclear. Thus, the transient hemodynamic response that occurs with hypoxic exposure may provide performance benefits up to the time of diminished mitochondrial function.

Oxidative Stress:
The collective effects of these data strongly suggest that the exercise stimulus elicited an oxidative stress response that became evident during the recovery period. The interesting discovery is that Hypoxic Recovery appeared to blunt many of these findings as compared to the Normoxic Recovery.

Examination of the data does raise questions about why some of the typical post exercise findings were not observed in the current samples. However, some of the expected common

post exercise increases in these markers were not statistically significant despite trends. Some of these findings may be related to hydration status of the subjects during the recovery period or the lower exercise intensity selected for year 3.

Given the influence of redox changes on adaptations to exercise, the decreased oxidative stress that we observed may provide the mechanism by which mitochondrial stimuli are diminished. Thus, oxidative stress is among the most potent stimuli for mitochondrial adaptations and our observations indicate that during hypoxic recovery this adaptive response does not continue to increase in a linear manner.

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Findings from year 3 – Field Study Series

INTRODUCTION

Wildland firefighters (WLFFs) are subjected to a multitude of environmental and physical demands on a day-to-day basis, and must be capable of responding quickly to contain wildfires. High energy (17.5 ± 6.9 MJ·d^{-1}) and fluid demands (6.7 ± 1.4 L·d^{-1}) result from hours of physical labor in hot environments in an effort to contain wildland fires (Ruby et al., 2003; Ruby et al., 2002). These firefighters work long hours on a variety of terrain and fuel types, leading to physical and mental exhaustion in an environment where awareness is crucial to maintain safety (Petersen et al., 2010).

Wildland firefighters respond to their stressful environment by altering their behavior and/or eating habits in response to any increase in energy expenditure during sustained strenuous work in heat (Gun and Budd, 1995). During tasks that may be shorter in duration, wildland fire crews are more likely to work harder compared to working longer shifts (Rodriguez-Marroyo et al., 2012). As a result of these extended work periods, the majority of the self-paced activity is comprised of sedentary or light work intensities (Montain et al., 2008). Firefighters work to accomplish specific, time-oriented tasks, resulting in irregularly scheduled breaks. This makes it difficult to implement a structured eating and drinking program, despite research indicating that eating regularly increases work output 10-20% (Cuddy et al., 2011; Montain et al., 2008).

Firefighters self-regulate body temperature over time when working on the fire line by altering their work strategies (Rodríguez-Marroyo et al., 2012). Direct, indirect, and mixed attack strategies of wildfire suppression increase core body temperatures above resting values, though each attack strategy results in similar elevations in core temperature (Rodríguez-Marroyo et al., 2011). Additionally, regardless of beverage type and volume consumed, wildland firefighters exhibited similar thermoregulatory responses resulting from well-balanced self-regulation (Cuddy et al., 2008a). Heart rate during wildfire suppression has shown a large degree of individual variability, suggesting the need for further studies to improve current thermoregulatory models (Yokota et al., 2012).

Thermoregulation is challenged with high ambient and radiant temperatures. When combined with high metabolic demand, the homeostatic systems of the body can be overwhelmed and lead to heat injury (Cheuvront et al., 2010). Hot environmental conditions also prevent significant recovery from arduous labor. This can lead to fatalities as a result of rigorous occupational tasks and exposure to the elements even if emergency procedures are conducted optimally for the situation (Cuddy and Ruby, 2011; NIOSH, 2012). Wildland

firefighters are continually encouraged to aggressively increase fluid intake during extended shifts. However, even with adequate hydration, high work intensity and high thermal load can result in heat exhaustion during wildland fire suppression (Cuddy and Ruby, 2011).

Substantial demand is placed on these occupational athletes during the tasks of their job as they seek to meet energy and fluid demands on a daily basis. Daily energy expenditure is often nearly double typical population norms as crews are deployed for shifts often lasting 16 hours. Self-selected patterns of physical activity during wildfire suppression play a large role in the degree of effect on firefighters where each task has a large degree of variability. The purpose of this investigation is to characterize the effects of self-selected work activity on energy expenditure, water turnover, and thermal strain during arduous wildland fire suppression. A secondary aim was to contrast the current data with data collected 15 years ago using similar methods to determine if the job demands have changed.

METHODS
Design
Participants (N=15) were recruited from two separate Type I Interagency Hot Shot fire crews and monitored for 3 days during the summer of 2012. Prior to participation, subjects provided informed consent by signing a university approved institutional review board consent form. Upon enrollment, participants arrived to the mobile laboratory in the PM prior to beginning their work shift the next day. Upon arrival, participants were weighed in the nude, verbally provided their height, and were provided with an oral dose of tracer water ($^2H_2^{18}O$).

Each day during enrollment in the study, participants arrived to the mobile laboratory in the early morning, submitted a nude body weight, ingested a disposable temperature transmitter pill (Jonah™ capsule, Vitalsense®, Mini Mitter, Bend, OR), provided a urine sample, and were equipped with the Hidalgo Equivital™ EQO2 LifeMonitor (Hidalgo Limited, Cambridge, UK) and an ActiCal activity monitor (Mini Mitter, Bend, OR). After being equipped with the sensors, participants went to work, which involved activities such as hiking, line digging, laying hose, chain sawing, clearing brush, lookout, and scouting. Each participant wore and carried traditional WLFF gear including: Nomex long-sleeved shirt and pants, mid-calf leather logging boots, a 100% cotton short-sleeve undershirt, leather gloves, hard hat, and a 12 – 20 kg pack. Work shifts (excluding drive time) averaged 11.4 ± 0.7 hours in duration. Participants reported to the mobile laboratory following the work shift, were weighed, and returned the monitors.

Energy Expenditure and Water Turnover
Doubly labeled water On the night before the three work days, participants consumed an oral dose of $^2H_2^{18}O$ (100 g; 1.82 g ^{18}O per kg total body water, 0.13 g$^2H_2$ per kg total body water). The dosing procedures and analysis were similar to previous work completed by this laboratory (Cuddy et al., 2008b; Ruby et al., 2003; Ruby et al., 2002). Briefly, urine samples were collected at the following time points: immediately prior to ingesting the dose, AM second voids on days 1, 2, 3, and 4, and PM voids on days 1, 2, and 3. On the first night, overnight and first void urine volumes were collected and measured. The isotopic enrichments of urines collected on each AM and PM were used to determine elimination rates, water turnover, and CO_2 production. Isotope enrichments were measured by isotope

ratio mass spectrometry. Total body water was estimated based on body weight changes relative to the initial time of isotopic dosing and the TBW/body weight relationship established at that time. Again, for a more detailed report of these procedures, see the following references (Cuddy et al., 2008b; Ruby et al., 2003; Ruby et al., 2002).

Weight
Participants were weighed in the nude using a calibrated scale (*Ohaus CW-11*, Pinebrook, NJ).

Ingestible Sensor
Researchers initialized a Jonah™ Ingestible Sensor (Mini Mitter, Bend, OR) prior to participant arrival to the lab. Participants ingested the sensor prior to eating breakfast. Researchers monitored participants while they were at the laboratory to ensure it passed from the stomach into the intestines. For analysis, random low temperature data points less than 36.5°C were dropped.

Hidalgo Equivital™ SDK Physiological Monitor
Participants were equipped with the Hidalgo Equivital™ EQO2 LifeMonitor physiological monitor according to manufacturer's directions. The sensor measured heart rate, respiration rate, skin temperature, body motion, and body position. The system has been certified by the Food and Drug Administration (FDA). The system was checked in real time on a computer using Hidalgo software and Bluetooth technology to ensure proper function. Participants wore the monitor for the entire work shift. Data were downloaded onto a computer and converted into Excel files for data analysis. Due to technical difficulties with the monitor (primarily battery failure in the ingestible sensor), complete HR, core and skin temperature data for the entire workshift was collected on 29 of the 45 "man-days." Thus, direct day to day comparisons were not possible to analyze similar to other dependent variables, so these variables are expressed as descriptive data to characterize physiological strain patterns during these days of wildland fire suppression. From the heart rate and core temperature data, the Physiological Strain Index (PSI) score was computed based on research by Moran et al. (Moran et al., 1998).

Activity Monitor
Activity counts were obtained using ActiCal® activity monitors (MiniMitter, Bend, OR). The monitors were initialized and distributed to crew members to determine activity counts during one day of firefighting. Researchers placed ActiCals® in the left chest pocket of the Nomex fire shirt as previously indicated (Cuddy et al., 2007). For protection, stability, and in order to keep the unit in a secure position, each monitor was secured in a white foam core square (~7.6 cm x 7.6 cm). This location was chosen due to the amount of upper body movement associated with WLFF. Activity counts were averaged into one-hour intervals and were expressed in counts·min^{-1}.

Diet and Activity Log
Participants were allowed to eat and drink *ad libitum* throughout the day. Participants recorded work tasks throughout the work shift and the associated rating of perceived exertion (Borg 6-20 scale), and the times associated with these jobs. At the end of the work shift,

researchers reviewed the logs with each individual participant to ensure accuracy.

Statistics

Differences in body weight and mean activity counts across the 3 day study were analyzed using a one-way ANOVA. Differences in rating of perceived exertion and time spent in different activity intensities were analyzed using two-way repeated measures ANOVA. Differences for TEE and rH_2O between data from 1997-98 vs 2012 were analyzed using a paired samples t-test. Body weight differences between data from 1997-98 vs 2012 were analyzed using a 2 x 2 mixed design ANOVA with repeated measures. All analysis was performed using SPSS for Windows Version 13 (Chicago, IL). A probability of Type 1 error less than 5% was considered significant ($p < 0.05$). Data are reported as mean ± standard deviation.

RESULTS

Body Weight

There was no change in body weight across the 3 day data collection period (77.3 ± 8.3, 76.9 ± 8.3, 77.5 ± 8.6, and 77.0 ± 8.9 for mornings 1, 2, 3, and 4, respectively, p = 0.12).

Heart Rate, Core and Skin Temperature

See Table 1 for a complete profile of heart rate, core and skin temperature, and PSI over the course of 3 days of wildland fire suppression.

Activity

Activity was higher on Day 1 compared to Days 2 and 3, and Day 2 was higher than Day 3 (See Figure 1). Participants spent less time sedentary on Day 1 compared to Days 2 and 3, and spent more time doing moderate/vigorous work on Day 1 compared to Days 2 and 3, and more time doing moderate/vigorous work on Day 2 compared to Day 3 (See Figure 2). On day 2 and 3 participants spent more time sedentary compared to light and moderate/vigorous, and more time doing light compared to moderate/vigorous.

Rating of Perceived Exertion

Rating of perceived exertion was lower on Day 2 compared to Days 1 and 3 during 1200-1400, 1400-1600, and 1600-1800, interaction effect, $p < 0.05$ (See Figure 3). It was lower on Day 2 compared to Day 3 at 1000-1200, but higher compared to Day 1. Day 1 was lower than Day 3 from 1000-1200, but higher than Day 3 from 1200-1400. Though different at a couple time points in the middle of the workshift, days 1 and 3 have a similar mean RPE for the entire workshift, 11.4 ± 2.2 vs 11.9 ± 1.4, respectively.

Total Energy Expenditure and Total Body Water Turnover

The mean TEE was 19.1 ± 3.9 $MJ·d^{-1}$ and the mean rH_2O was 9.5 ± 1.7 $L·d^{-1}$. Individual subject data, as well as means for all EE variables, can be seen in Table 2. A retrospective analysis of the current data set compared to the 2002 wildland fire paper by Ruby et al. (Ruby et al., 2002)can be seen in Table 3. Briefly, there was no difference in TEE, while rH_2O was higher in 2012 compared to 1997-98, $p < 0.05$.

Ambient Conditions

The ambient weather conditions were reported using the *Fort Collins Weather Station Data Access* (http://ccc.atmos.colostate.edu/~autowx/fclwx_access.php) for the dates and time of day associated with data collection.

Table 1. Physiological strain indices during 3 days of wildland fire suppression. Data is expressed as percentage of time (mean ± stdev) during the workshift that was spent within the designated range. N = 29 "man-days."

| Heart Rate	<100	100-120	120-140	140-160	160-180	≥ 180
	37 ± 19%	25 ± 12%	20 ± 8%	12 ± 8%	4 ± 5%	1 ± 2%
Core	**<37.5**	**37.5-38.0**	**38.0-38.5**	**38.5-39.0**	**39.0-39.5**	**≥ 39.5**
	34 ± 24%	48 ± 19%	17 ± 15%	1 ± 3%	0 ± 0%	0 ± 0%
Skin	**<30**	**30-32**	**32-34**	**34-36**	**36-38**	**≥ 38**
	2 ± 4 %	13 ± 14%	27 ± 18%	44 ± 17%	14 ± 14%	0 ± 0%
PSI	**Rest**	**1-2**	**3-4**	**5-6**	**7-8**	**9-10+**
	9 ± 13%	33 ± 10%	38 ± 9%	17 ± 7%	2 ± 2%	0 ± 0%
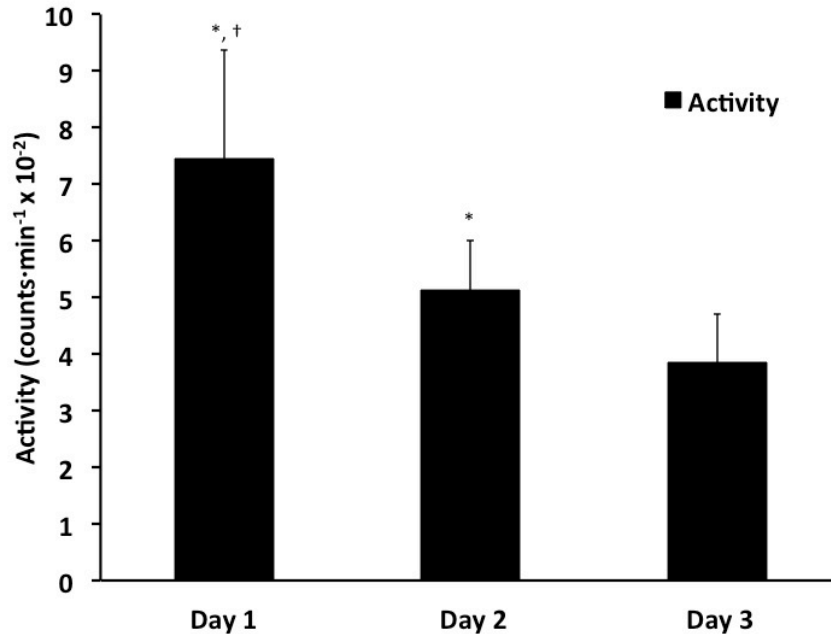
Figure 1. Activity patterns during 3 days of wildland fire suppression. * higher compared to Day 3, $p<0.05$; † higher compared to Day 2, $p<0.05$.
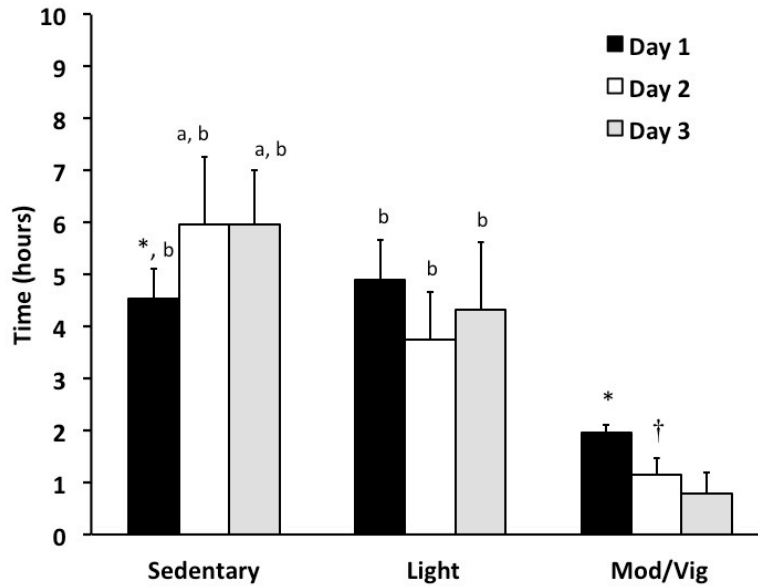
Figure 2. Time spent at different intensities during wildland fire suppression over 3 days of work. * different compared to Days 2 and 3, interaction effect, $p < 0.05$. † different compared to Day 3, interaction effect, $p < 0.05$. *a* different compared to light and moderate/vigorous within the day, interaction effect, $p < 0.05$. *b* different compared to moderate/vigorous within the day, interaction effect, $p < 0.05$
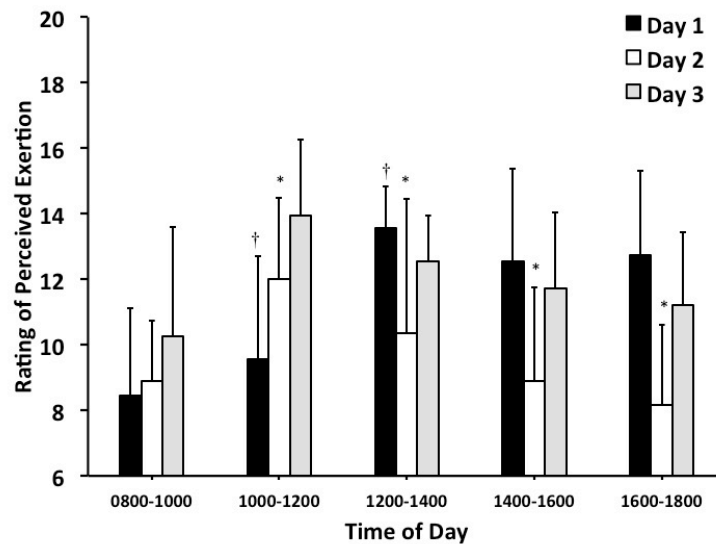
Figure 3. Rating of perceived exertion across the workshift and over 3 days of wildland fire suppression. * different compared to Days 1 and 3, interaction effect, $p < 0.05$. † different compared to Day 3, interaction effect, $p < 0.05$.

Table 2. Individual descriptive, energy expenditure, and water turnover values during wildland fire suppression efforts calculated from 1-3 d elimination rates.

Sex	Age (yrs)	Ht (cm)	Exp (yrs)	TEE MJ·d^{-1} (kcal·d^{-1})	EEA MJ·d^{-1} (kcal·d^{-1})	TEE xBMR	rH$_2$O L·d^{-1}	BW (kg) Pre	BW (kg) Post
M	25	180	6	18.3 (4383)	9.1 (2168)	2.5	9.29	78.6	77.0
M	25	180	7	18.7 (4461)	10.2 (2428)	2.8	9.83	70.4	69.0
M	28	183	7	19.8 † (4728 †)	9.9 † (2369 †)	2.5 †	11.75	85.0	83.1
F	25	175	8	15.9 (3812)	8.4 (1998)	2.7	8.55	63.7	61.5
F	26	170	7	13.4 (3197)	5.8 (1378)	2.1	7.11	66.1	64.9
M	26	178	5	21.5 (5130)	11.7 (2808)	2.8	9.51	77.0	76.2
M	26	175	4	12.3 (2946)	3.8 (896)	1.7	11.74	76.8	75.7
M	23	188	4	23.5 (5624)	13.2 (3156)	3.0	11.53	91.6	94.4
M	25	178	5	20.0 (4774)	10.8 (2584)	2.8	8.14	73.7	72.7
M	22	180	4	21.5 (5131)	11.7 (2792)	2.8	7.87	80.2	78.4
M	23	178	5	19.3 (4605)	9.9 (2373)	2.6	11.35	87.4	86.5
M	21	183	4	18.3 (4369)	8.9 (2139)	2.4	9.45	81.5	79.5
M	26	178	7	24.3 (5811)	15.0 (3589)	3.5	10.39	76.5	75.3
M	29	193	9	25.5 (6083)	14.5 (3475)	3.0	9.74	93.1	89.8
F	33	170	11	13.8 (3290)	6.5 (1562)	2.4	6.05	72.3	70.8
Mean	**26 ± 3**	**179 ± 6**	**6 ± 2**	**19.1 ± 3.9 (4556 ± 943)**	**10.0 ± 3.1 (2381 ± 746)**	**2.6 ± 0.4**	**9.49 ± 1.73**	**78.3 ± 8.6***	**77.0 ± 8.9**

different compared to post, p<0.05
† *data is representative of 2 days of wildland fire suppression*

DISCUSSION

During wildland fire suppression many factors challenge the maintenance of optimal physical performance and well-being, such as imperfect sleeping situations, irregular dietary patterns, long work shifts, repeated exposure to high ambient temperatures and smoke, and difficulty implementing quality hygiene practices (Aisbett et al., 2012). This is the first study to comprehensively assess the effect of work activity patterns on a host of physiological and thermoregulatory parameters during wildland fire suppression in the western United States. The purpose of this investigation was to characterize the effects of work activity on energy expenditure, water turnover, and thermal strain during wildland fire suppression by wildfire hand crews. The current data set suggests WLFFs expend 19.1 ± 3.9 MJ·d^{-1} (4556 ± 943 kcal·d^{-1}), have an rH$_2$O of 9.5 ± 1.7 L·d^{-1}, and an overall physiological strain characterized as

"Little" to "Low" (Moran et al., 1998), which comprised 81 ± 14% of the total work day. A secondary aim was to contrast the current data with data collected 15 years ago using similar methods to determine if the job demands have changed. These data suggest the total energy expenditure (TEE) during wildland fire suppression has not changed in 15 years, despite a higher total body water turnover (rH_2O) of 2.5 L·d^{-1} in 2012 compared to 1997-98.

In the current study, wildland firefighters averaged 745 ± 191, 512 ± 89, and 385 ± 86 counts·min^{-1} across the 3 days of fire suppression efforts. Since previous research during wildland fire suppression has demonstrated mean daily activity levels between 175 and 724 counts·min^{-1} (Cuddy et al., 2007; Cuddy et al., 2008a; Cuddy et al., 2011; Montain et al., 2008), the varied daily activity patterns in the current study are within the range of expected activity patterns during the job, with day 1 being the highest daily average collected to date (8 of 15 participants averaged > 850 counts·min^{-1}). The WLFFs in the current study spent 49 ± 8%, 39 ± 6%, and 12 ± 2% in the sedentary, light, and moderate-vigorous intensity categories over the 3 days of fire suppression efforts, a shift from the typical percentages seen in recent papers. Previous work with WLFFs has revealed greater time spent in the sedentary category during the workshift (61-66% (Cuddy et al., 2007) and 74% (Cuddy et al., 2011) of time) and less time spent in the moderate-vigorous categories (0-7% (Cuddy et al., 2007) and 5% (Cuddy et al., 2011)) compared with the current data set. In short, though activity counts in the current study represented typical patterns seen with this population, considerably less time was spent being sedentary and more time was spent moving.

Similar to previous findings, the HR and core temperatures of wildland firefighters in the current study did not reflect high levels of cardiovascular or thermal strain (Rodriguez-Marroyo et al., 2012). Sixty three percent of the time WLFF exhibited HR values less than 120 BPM (daily average 112 ± 13 bpm), while 82% of the time core temperature was below 38.0°C. Rodriquez et al. found that for wildfires above 5 h in duration, mean core temperature was 37.8 ± 0.3°C, similar to the current study, where mean core temperature was 37.6 ± 0.2°C during workshifts lasting 11.4 ± 0.7 h. Further, Rodriquez reported mean PSI of 3.9 ± 0.5, while in the current study it was 3.3 ± 1.0. WLFFs have practical learned strategies to alleviate heat stress, particularly: avoiding unnecessary heat, self-pacing, evaporating sweat, and replacing sweat losses by drinking (Budd, 2001). Additionally, Schlader et al. showed that when allowed to self-pace during exercise in the heat, participants chose a lower exercise intensity, which decreased metabolic heat production and induced lower levels of thermoregulatory strain, providing a more compensable situation (Schlader et al., 2011). It is likely WLFFs moderate their work efforts to maintain safe levels of thermal and cardiovascular strain, provided they know they have extended shifts (10-16 h). However, it is unclear if this is more prevalent in Type 1 crews (Hotshot) vs. less experienced Type 2 crews, as it may be that the level of experience lends itself to smarter work behaviors.

Although HR, core temperature, and PSI appear low, the thermal demand from the environment (ambient 27.4 ± 3.9°C; range 20.5 ± 1.3-30.6 ± 4.1°C) coupled with wearing personal protective equipment (Nomex long-sleeved shirt and pants, mid-calf leather logging boots, a 100% cotton short-sleeve undershirt, leather gloves, hard hat, and a 12 – 20 kg pack) creates a challenging microenvironment, as evidenced by chest skin temperatures. In this study, 42% of the time skin temperature was below 34.0°C, while 58% of the time skin

temperature was greater than 34.0°C, of which 14 ± 14% of time skin temperature was between 36.0-38.0°C, approaching the upper end of skin temperatures where a human can still dissipate heat to the environment. To compare with laboratory studies, Kenefick et al. observed skin temperatures of 35.9 ± 0.4 and 36.3 ± 0.6°C during exercise in ambient conditions of 40°C for 45 min (Kenefick et al., 2010). Sawka reports impaired submaximal performance with increasing skin temperatures, demonstrating the narrow gradient between skin and core temperature as skin temperatures approach 34.0 and 36.0°C with core temperatures around 38.0°C (Sawka et al., 2012). Buller demonstrates that above skin temperatures of 35.0°C, exercising humans are likely to reach PSI values of 7.5 and greater if heart rate rises, particularly above approximately 160 bpm. Perhaps the self-pacing practices of experienced WLFF has a strong relationship to internal feedback mechanisms related to elevated skin temperatures; WLFF will keep cardiovascular strain low (reduced work output) to compensate for the pressing ambient conditions, thus maintaining an overall lower thermal strain.

In 2002, Ruby et al. published a paper to assess the energy expenditure demands of wildfire suppression, concluding that the mean energy expenditure was 12-26 MJ·day^{-1} (2868 – 6214 kCal·day^{-1}) (Ruby et al., 2002). Assuming a 16-hour work shift, this amounts to 219 ± 57 kcal·hr^{-1}, while a 12-hour work shift would equate to 264 ± 73 kcal·hr^{-1}. The primary factors affecting energy expenditure during wildland fire suppression are: work assignment, self-selected work intensity, and the location of the fire (Ruby et al., 2002). Additionally, body size (weight), fat-free mass, pack weight, and gear carried affect it as well. In the 15 years since this data was collected (summers of 1997-98), firefighters anecdotally claim the job has changed substantially, with suppression efforts being much more conservative to minimize risk. To test this hypothesis, energy expenditure and water turnover was measured in the current study with the same methods used 15 years ago. In the current study, the mean TEE was 19.1 ± 3.9 MJ·d^{-1} (4556 ± 943 kcal·d^{-1}), while the previous manuscript reported mean TEE as 17.5 ± 4.1 MJ·d^{-1} (4177 ± 979 kcal·d^{-1}). There was no difference between the TEE, however, water turnover was higher in 2012. In the current study, mean rH$_2$O was 9.5 ± 1.7 L·d^{-1}, while in the previous paper it was 7.0 ± 1.7 L·d^{-1}, a mean difference of 2.5 L·d^{-1}. Ambient conditions during the current study were cooler compared to the 1997-98 data, with an average high temperature of 30.6 ± 4.1°C compared to 35.3 ± 2.6°C, respectively.

This comparison for TEE and rH$_2$O should be approached with caution, since each study represents an incredibly small sample size compared to the number of wildland firefighters in the work force, though both studies collected data from experienced Type 1 Hotshot crews. Nonetheless, the elevated rH$_2$O in 2012 compared to 1997-'98 presents an interesting discussion point. During daily safety briefings at wildfires there is always a strong emphasis on aggressive fluid intake to prevent dehydration. Perhaps this ideology has influenced the drinking patterns of wildland firefighters, as previous studies (all between 1997 and 2007) show mean rH$_2$O values 7.0 ± 1.7 L·d^{-1}, 6.7 ± 1.4 L·d^{-1}, and 6.6 ± 2.1 L·d^{-1} across an N=59 in 3 separate studies (Montain et al., 2008; Ruby et al., 2003; Ruby et al., 2002). Although the observed increase in rH$_2$O may reflect an agency driven emphasis in aggressive fluid intake, it cannot be interpreted as an effective approach to reduce the potential for heat related illnesses, as a heat related illness was observed during wildland fire suppression efforts despite a high volume of drinking (Cuddy and Ruby, 2011).

While there is no doubt the environmental conditions are hostile during wildland fire suppression, the physiological data demonstrate that Type 1 Hotshot wildland firefighters do an adequate job of managing their environmental and work demands so that they do not induce consistently high cardiovascular and thermal strain. Despite sustaining relatively high skin temperatures throughout the workshift, WLFFs modulate their work activity to effectively compensate for the environmental conditions. The TEE in the current study suggests that the demands of the job are similar to 15 years ago, while the increased rH_2O may reflect a change in drinking habits by wildland firefighters.

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KEY RESEARCH ACCOMPLISHMENTS:

- All stated objectives in the statement of work have been accomplished.
- Multiple research presentations and manuscripts have been developed (see below).
- Additional research directions based on these findings have been developed.

REPORTABLE OUTCOMES:

- Research Abstracts

Small Differences in Exercise Intensity May Impact Muscle Glycogen Without Concurrent Changes in Metabolic Gene Expression.
Matt Heesch, Dustin Slivka, Charles Dumke, John Cuddy, Walter Hailes, and Brent Ruby

Intensity in exercise studies is often established relative to VO_2 peak. However, when there is an experimental manipulation of altitude, the intensity may be different depending on the altitude at which VO_2 peak is measured. **PURPOSE:** To determine the impact of different absolute intensities associated with 60% VO_2 peak, when VO_2 peak is measured at 975 m or at a simulated altitude of 3000 m on metabolic gene expression. **METHODS:** Twelve recreationally trained males (age 23.8 ± 3.8 y, mass 81.6 ± 11.6 kg, body fat 14.6 ± 7%) had their VO_2 peak measured on a cycle ergometer at 975 m and 3000 m. Participants then completed two trials consisting of cycling at 965 m elevation for 60 min at 60% VO_2 peak as measured at (A) 975 m and (B) 3000 m. Skeletal muscle biopsies were obtained from the *vastus lateralis* before exercise (PRE) and after 4 hours of recovery (POST4). Gene expression was measured using real-time RT PCR and expressed using the ΔΔ CT method. **RESULTS:** VO_2 peak was statistically similar when measured at 975 m and 3000 m (p=0.139; 4.24 ± 0.89 L·min^{-1} and 4.03 ± 0.60 L·min^{-1}). The absolute intensities were 174 ± 33 watts for trial A and 158 ± 23 watts for trial B (p>0.05). The VO_2 during trial A (2.48 ± 0.40 L·min^{-1}) was not significantly different than during trial B (2.43 ± 0.56 L·min^{-1}). Skeletal muscle glycogen was similar between trials at PRE but at was 37.6% higher in trial B at POST4 (p<0.05). There were no differences between trials for COX, HIF, PGC1, FIS, MFN, OPA, HK or PFK (p>0.05) gene expression. However, COX, HIF, PGC1, FIS, MFN, HK and PFK increased as a result of exercise regardless of trial (p<0.05) while OPA did not. **CONCLUSION:** These data indicate that small differences in exercise intensity created by completing initial VO_2 peak tests at 975 m versus 3000 m environment have an effect on muscle glycogen but not on select metabolic genes.

Dose-response of hypoxia on mitochondrial related gene expression

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Mitochondrial function is increased in repeated short term exposure to hypoxia. However, chronic hypoxia exposure has been shown to decrease mitochondrial function. It is unknown if a dose-response relationship between mitochondrial gene expression and magnitude of hypoxia impacts this paradox in mitochondrial function between acute and chronic hypoxic exposure. **PURPOSE:** To determine the mitochondrial related gene response to incremental levels of hypoxia. **METHODS:** Recreationally-trained male cyclists (n = 10, age 24 ± 4 y, height 183 ± 5 cm, weight 86 ± 14 kg, 17 ± 8% body fat, VO_2 peak 4.0 ± 0.5 L · min^{-1}, W_{max} 287 ± 42 W) completed a 60-minute ride at 70% of W_{max} at an altitude of 975 m, followed by 6 h of recovery at four different simulated altitudes (0 m, 1667 m, 3333 m, or 5000 m). Blood O_2 saturation was measured via pulse oximetry every hour during the 6 h recovery period. Muscle biopsies were obtained from the *vastus lateralis* pre- and 6 h post-exercise for analysis of mitochondrial related gene expression. **RESULTS:** Blood O_2 saturation decreased with each increase in simulated altitude during recovery (0 m: 98 ± 1%; 1667 m: 94 ± 1%; 3333 m: 90 ± 1%; 5000 m: 79 ± 2%; $p < 0.05$). Expression of PGC-1α, HK, and SOD increased significantly with exercise ($p < 0.05$), but were not different between trials. There was a tendency for expression of HIF-2α to increase with exercise, although this did not reach statistical significance ($p = 0.089$). There were no differences in HIF-1α, PFK, FIS-1, MFN2, OPA-1, HO, or NFE2L2 with exercise or between trials ($p > 0.05$). **CONCLUSION:** These data demonstrate no dose-response relationship between magnitude of hypoxic exposure and mitochondrial gene expression. Therefore, the paradox of mitochondrial function in response to acute and chronic exposure to hypoxia cannot be explained by the magnitude of hypoxia.

Impact of Hypoxia on Metabolic Gene Expression

Dustin Slivka, Matt Heesch, Charles Dumke FACSM, John Cuddy, Walter Hailes, and Brent Ruby FACSM

Hypoxia may be a potent stimulator for metabolic adaptation. However, the metabolic mechanism is not well characterized. **PURPOSE:** To determine the impact of acute hypoxia on the expression of genes associated with anaerobic and aerobic metabolism after exercise. **METHODS:** Twelve recreationally trained males (age 23.8 ± 3.8 y, mass 81.6 ± 11.6 kg, body fat 14.6 ± 7%, VO$_2$ peak 4.03 ± 0.60 L·min^{-1}) exercised at an absolute workload associated with 60% VO$_2$ peak (158 ± 23 watts) in an altitude chamber that simulated the hypoxia associated with altitudes of 975 m (LOW) and 3000 m (HIGH) in a randomized order. Skeletal muscle biopsies were obtained from the vastus lateralis before exercise and after 4 hours of recovery. Gene expression was measured using real-time RT PCR and expressed using the ΔΔ CT method. **RESULTS:** VO$_2$ was not different during exercise between LOW and HIGH (2.43 ± 0.56 L·min^{-1} and 2.42 ± 0.35 L·min^{-1}, respectively). Blood oxygen saturation was similar before each trial (LOW, 97.5 ± 0.9 %; HIGH, 97.7 ± 1.3 %) but was higher in LOW than HIGH during exercise ($p < 0.05$; 97.0 ± 0.8 % and 91.1 ± 1.5 %, respectively) and recovery ($p < 0.05$; 98.0 ± 0.5 % and 94.3 ± 1.4 %, respectively). Muscle glycogen reduced as a result of exercise ($p < 0.05$) but was not different between trials. There were no differences between trials for COX, HIF, PGC1, FIS, MFN, OPA, HK or PFK ($p>0.05$) gene expression. However, COX, HIF, PGC1, FIS, MFN, HK and PFK increased as a result of exercise regardless of trial ($p < 0.05$) while OPA did not. **CONCLUSIONS:** These data indicate no differences in select metabolic gene expression between acute exercise at 975 m and 3000 m when absolute intensity is held constant despite differences in blood oxygen saturation.

Mitochondrial Related Gene Expression is Suppressed After Simulated High Altitude Exposure.

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Short term exposure to altitude / hypoxia (train high / live low) has demonstrated an increase in mitochondrial function whereas chronic exposure (train high / live high) has shown a decrease in mitochondrial function. **PURPOSE:** To determine the acute gene response after exercise when exposed to simulated high altitude during recovery. **METHODS:** Ten recreationally active males (25 ± 2 yrs, 78.9 ±8.6 kg, 178 ± 8 cm, 13.1 ± 4.5% fat, 4.3 ± 0.6 L·min^{-1} VO$_2$ max) cycled for 90 minutes in laboratory conditions and then either recovered for 6 hours in laboratory conditions (975 m; normoxia) or at a high simulated high altitude (5000 m; hypoxia). Skeletal muscle biopsies from the vastus lateralis were obtained before exercise, after exercise, and 6 hours after exercise for the measurement of metabolic gene expression and muscle glycogen. Blood oxygen saturation was measured via pulse oximetry before exercise, after exercise, and during recovery. Participants received a liquid carbohydrate beverage immediately after exercise (1.2 g·kg^{-1}) and solid feedings (1.28 g · kg^{-1} carbohydrate, 0.15 g · kg^{-1} fat, and 0.29 g · kg^{-1} protein) at 2 and 4 hours into recovery. **RESULTS:** Blood oxygen saturation was lower during hypoxia trials than normoxia trials (p < 0.05). Muscle glycogen decreased with exercise (61 ± 13%, p < 0.05) and increased with recovery (78 ± 35%, p < 0.05) with no difference between trials (p > 0.05). HIF1α, HIF2α, OPA1, MFN2, NRF2, SOD, COX, and PGC1α gene expression were suppressed after altitude exposure (p < 0.05) while FIS1, HO1, PFK, and HK were unaffected by altitude exposure (p > 0.05). **CONCLUSION:** High altitude exposure during recovery from exercise inhibits gene expression associated with mitochondrial development without affecting muscle glycogen re-synthesis. These data may explain the mechanism by which mitochondrial function is reduced after extended stays at altitude.

Effect of Acute Hypoxia on Exercise-Induced Blood Oxidative Stress

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Hypoxia has been characterized by decrement in exercise performance or decreased maximal workload. Exercise and exposure to altitude have been shown to elicit cellular hypoxia independently and combined. Similarly, these stimuli also elicit perturbations in redox balance. **PURPOSE**: The purpose of this study was to examine the effect of hypobaric chamber-simulated-hypoxia on exercise induced blood oxidative stress at variable relative intensities. **METHODS**: Physically active males (n=12) completed 2 graded exercise tests on an electronically braked cycle ergometer starting at 95 W, increasing 35 W every 3 min until volitional fatigue or cadence < 50 rpm. VO2peak and Wmax were measured at low altitude (975m, "lowALT") and high altitude (3000m, "highALT") simulated in a hypobaric chamber. Wmax at both altitudes was used to program workloads for subsequent trials. In a randomized counterbalanced cross-over design, subjects completed 3, 60 min exercise bouts at combinations of lowALT or highALT at workloads corresponding to 60 percent VO2peak measured at 975m or 3000m (lowINT and highINT, respectively). The conditions were paired: lowALT:highINT, lowALT:lowINT, and highALT:lowINT. Subjects remained in the ambient altitude for 4 hr recovery. Blood was drawn from the antecubital vein pre-, 0, 2 and 4 hours post exercise and analyzed for biochemical markers of oxidative stress. Samples were assayed for Ferric Reducing Ability of Plasma (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), Lipid Hydroperoxides (LOOH) and Protein Carbonyls (PCs). Results were adjusted for plasma volume shift and were analyzed with repeated measures ANOVA, significance set at $p < 0.05$. **RESULTS**: LOOH were elevated in highALT:lowINT group immediately post and 2HR post. highALT:lowINT was significantly elevated from lowALT:highINT at 2HR post. Main effects were seen in FRAP (for TRIAL, lowALT:highINT vs. lowALT:lowINT), PCs (for TIME), LOOH (for TRIAL and TIME) and TEAC (for TIME). **CONCLUSIONS**: These results suggest that acute hypoxia induced by hypobaric elevation increases the oxidative stress response during exercise.

EFFECT OF HYPOXIC RECOVERY POST-EXERCISE ON BLOOD OXIDATIVE STRESS MARKERS

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Exercise combined with high altitude exposure causes decrements to exercise performance and alters blood oxidative stress responses. Oxidative stress is typically quantified during exercise recovery but the impact of the ambient conditions on post exercise oxidative stress outcomes is unknown. In particular, the influence of hypoxia during exercise recovery on blood redox balance remains undetermined. **PURPOSE:** This study investigated effects of a hypobaric-hypoxia exercise recovery on acute post exercise blood oxidative stress. **METHODS:** Physically active males (n=12) performed a bout of cycle ergometer exercise on two occasions consisting of 10 intervals (2 minutes at 80% VO2max and 4 min at 50% VO2max) followed by 8 min at 60% VO2max and 12 min at 50% VO2max. Subjects recovered for 6 hours at either 975 m or 5000 m (hypobaric chamber) in a randomized counter-balanced cross-over design. O2 saturation was monitored during exercise and recovery via finger pulse oximetery. Blood samples were obtained pre- (PRE), post- (POST), 2 hrs post- (2HR), 4 hrs post- (4HR), and 6 hrs post-exercise (6HR). Samples were assayed for Ferric Reducing Ability of Plasma (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Protein Carbonyls (PCs). Results were adjusted for plasma volume shifts and analyzed with repeated measures ANOVA, significance set at $p \leq 0.05$. **RESULTS:** Results showed modest blood oxidative stress responses for PCs, TEAC, and FRAP. PCs were not significantly altered between trials, but a time main effect (p=0.044) indicated an exercise induced blood oxidative stress response. Plasma TEAC values were significantly elevated in the normoxic but not hypoxic recovery (p=0.044, 1Hr, 2Hr, and 6Hr). Plasma FRAP values were higher 6Hr (p=0.045) in normoxic versus hypoxic recovery. Analysis of the percent change in plasma FRAP indicated a main effect of time (p=0.032) and a significant decrease at the 6Hr hypoxic recovery time point (p=0.045) versus normoxic recovery. **CONCLUSION:** These data indicate the exercise stimulus elicited an oxidative stress response that was evident in blood parameter changes during recovery. Hypoxic exercise recovery blunted these outcomes as compared to the normoxic recovery.

RECOVERY AT VARYING ALTITUDES AND BLOOD OXIDATIVE STRESS

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Purpose: Exercise at a moderate altitude elicits blood oxidative stress while exercise recovery at an altitude of 5000m attenuates this response as compared to normoxia. Since the thresholds of these phenomena remain unknown, this study examined exercise recovery affects at 4 simulated altitudes on blood oxidative stress. **Methods**: Active males between the ages of 18-40 (n=12) performed cycle ergometery for 60 minutes at 70% VO2 max in the laboratory base altitude of 975 meters. In a randomized counter-balanced crossover design, subjects recovered for 6 hrs at 0 m, 1667 m, 3333 m, and 5000m in a normobaric hypoxia chamber. Pulse oximetry was used to measure oxygen saturation throughout the exercise and recovery periods. Blood samples obtained pre-, post-, 1 hour post-, and 5 hours post-exercise were assayed for the following antioxidant and oxidative stress biomarkers: ferric reducing antioxidant plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), uric acid (UA), and lipid hydroperoxides (LOOH). **Results:** Pulse oximetry data during exercise were statistically similar for all trials, while trial-dependent differences in blood oxygen saturation existed for the duration of exercise recovery ($p<0.05$). Assay results indicated a time-dependent blood oxidative stress occurred following exercise for all variables ($P<0.05$), but the two highest recovery altitudes (3333m & 5000m) partially attenuated this response for LOOH ($P<0.000$). **Conclusion:** These data suggest exercise recovery at varying altitudes may result in alteration of redox balance and blood oxidative stress markers.

Research Manuscripts in Preparation or Recently Published

DETERMINING EXPERIMENTAL EXERCISE INTENSITY BASED ON EXERCISE CAPACITY TESTS CONDUCTED IN HYPOXIC AND NORMOXIC ENVIRONMENTS

Dustin R. Slivka, Matthew W.S. Heesch, Charles L. Dumke, John S. Cuddy, Walter S. Hailes, and Brent C. Ruby

Journal: Wilderness and Environmental Medicine, In Review

HUMAN SKELETAL MUSCLE MRNA RESPONSE TO A SINGLE HYPOXIC EXERCISE BOUT

Dustin R. Slivka, Matthew W.S. Heesch, Charles L. Dumke, John S. Cuddy, Walter S. Hailes, and Brent C. Ruby

Journal: Wilderness and Environmental Medicine, In Review

ACUTE HYPOXIA AND EXERCISE-INDUCED BLOOD OXIDATIVE STRESS

Graham McGinnis, Brian Kliszczewiscz, Matthew Barberio, Christopher Ballmann, Bridget Peters, Dustin Slivka, Charles Dumke, John Cuddy, Walter Hailes, Brent Ruby and John Quindry

Journal: International Journal of Sports Nutrition and Exercise Metabolism, In Review

EXERCISE-INDUCED OXIDATIVE STRESS AND HYPOXIC EXERCISE RECOVERY

Christopher Ballmann ,Graham McGinnis, Bridget Peters, Dustin Slivka, John Cuddy, Walter Hailes, Charles Dumke, Brent Ruby and John Quindry

Journal: European Journal of Applied Physiology. 2014 Jan 3. [Epub ahead of print]

GRADED HYPOXIA AND BLOOD OXIDATIVE STRESS DURING EXERCISE RECOVERY

Bridget Peters, Christopher Ballmann ,Graham McGinnis, Haden Hyatt, Dustin Slivka, John Cuddy, Walter Hailes, Charles Dumke, Brent Ruby and John Quindry

Journal: International Journal of Sports Nutrition and Exercise Metabolism, In Preparation

WORK PATTERNS DICTATE ENERGY DEMANDS AND THERMAL STRAIN DURING WILDLAND FIREFIGHTING

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CONCLUSIONS

The purpose of this research determines how hypoxia interacts with exercise and recovery to yield various metabolic responses that may affect performance and performance at high-altitude critical to mission success. From study 1 and 2 of years 1 and 2, we noticed that exercise in hypoxic (3000 m) and normoxic (975 m) environments results in similar responses (glycolytic, metabolic, and mitochondrial morphology genes, and oxidative stress markers) when the exercise intensity is clamped relative to the specific environment (as a % of either hypoxic or normoxic peak power). Although there were subtle differences in the glycogen response to exercise/recovery, this is likely a function of the higher absolute work rate during the NN trial. In addition, we noted that gene expression of mitochondrial related genes was reduced after exercise as a function of recovery in an aggressive hypoxic environment.

From this, it may be speculated that a mild hypoxic stress may produce beneficial mitochondrial adaptations. In contrast, exposure to more extreme hypoxia may diminish the degree of mitochondrial adaptation. In contrast, in year three did not confirm these results, which may in part be related to the differences in exercise stimuli. Only under aggressive exercise of varied intensity did we notice a hypoxia driven reduction in mitochondrial gene expression. It is possible that the exercise intensity selected for year 3 (70% of max watts), was not high enough to denote hypoxic specific post-exercise response patterns.

Additionally, training in an aggressive hypoxic environment may negatively impact the muscle adaptive response (as noted in study 2, but not confirmed in study 3). The exact mechanisms associated with the paradox of increased mitochondrial function with repeated acute hypoxic exposure and decreased mitochondrial function with long term exposure remain unclear. Thus, the transient hemodynamic response that occurs with hypoxic exposure may provide performance benefits up to the time of diminished mitochondrial function.

The conclusions established from our aggressive field study clearly demonstrate that the occupational demands of the WLFF parallel those of the elite warfighter. More specifically, these data confirmed that instrumentation developed to monitor real-time hydration status should be questioned for this or similar field settings. Neither combination of physiological metrics we collected from mobile monitoring systems could account for the variance observed in 24-hour rates of water turnover and/or TEE. More importantly, mobile monitoring requires significant improvements in engineering, data capture and software development to be considered of practical significance under real-time field conditions. Data loss due to instrumentation failure was unacceptably high (approximating 30%). Secondarily, measures heat stress show promise for real-time scenarios if they are not reliant on measures of core temperature. Overall, our field study demonstrates that for physiological metrics to be of significant value to operators and/or commanders, they must provide meaningful, real-time information that can positively impact health (reduction of risk for heat related injury) or the overall mission. Without this real-time, practical impact, physiological monitoring in the operational environment simply adds complexity and unnecessary load carriage to the warfighter.